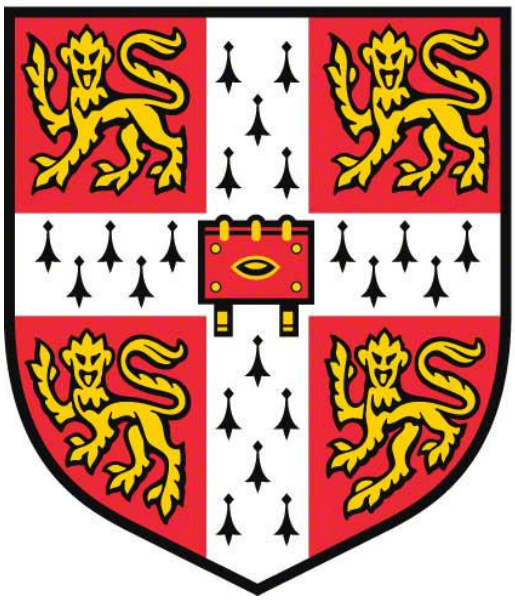
The role of ventrolateral prefrontal cortex in performance of spatial self-ordered response sequences in the marmoset



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This thesis is submitted for the degree of

Doctor of Philosophy

To my parents and my brother,

for always supporting me

Preface

The work presented in this thesis was carried out during October 2016 to September 2020 at the Department of Psychology, University of Cambridge, under the supervision of Professor Trevor W Robbins and Professor Angela C Roberts.

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the preface and specified in the text.

It is not substantially the same as any work that has already been submitted before for any degree or other qualification except as declared in the preface and specified in the text.

It does not exceed the prescribed word limit for the Degree Committee of Biology.

Acknowledgments

Firstly, I would like to express my sincerest gratitude to Professor Trevor Robbins and Professor Angela Roberts. You have supported me since day one of this PhD and without your support, none of this would have been possible. I have developed a lot under your mentorship! I also feel very grateful that you trusted me to pursue my experiments with independence. That independence would never have been possible without excellent training, guidance and support from Dr Nicole Horst during my time in the lab.

I am also very thankful for the help and support provided by all of my other colleagues at the Innes. Whether it was holding for an infusion, help monitoring during surgery or just an uplifting chat between testing, you have all been there for me. Special mention to Lisa and Zuzanna that started this journey at the same time as me, Shaun, for helping me with Kami, and Lauren, for always being a rock.

During my four years in the lab, I know my marmosets have always received excellent care by NACWO Mr Colin Windle and his team of technicians. I want to thank all of the technicians, not only for great care, but also for being able to flexibly work around my experimental requirements. I am also grateful for all assistance and guidance provided by NVS Dr Jo Keeley.

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I am also thankful for the support of friends during this PhD. The Friday pub crew core, Nicole, Ben, Laura and Chris have provided an excellent escape from lab life on Fridays. ‘Snillen’ have always been present and I am very happy for all evenings we have spent on discord and for your warm and embracing welcome when I have been back in Stockholm. My time in Cambridge have been enhanced by friends travelling from abroad to come and visit me, it really has meant a lot. An extra thank you to the friends that have bothered to come more than once: Rikard, Mille, Emil, Robin, Victor and Andreas.

I am also very fortunate to have had Emily by my side during this PhD. Thank you for all your support - my time with you has been and continues to be, wonderful!

Finally, thank you to my parents and my brother for encouraging me to pursue this PhD and for always supporting me unconditionally!

Abstract

The role of ventrolateral prefrontal cortex in performance of spatial self-ordered response sequences in the marmoset

Sebastian Folke Amandus Axelsson

The ventrolateral prefrontal cortex (vlPFC) in primates plays an important role in cognitive control and working memory, but as argued in the Introduction its contribution to those aspects of goal-directed behaviour such as planning and executing spatial response sequences requires further analysis, using more refined methods than have been employed hitherto.

These studies investigated the role of vlPFC in performance of self-ordered response sequences using intra-cerebral microinfusions of specific pharmacologic agents in the common marmoset. Following a description of the necessary methodology, including behavioural training and surgical details (Chapter 2), a causal role for vlPFC in performance of spatial-self ordered sequences was confirmed in Chapter 3 by demonstrating that local inactivation of vlPFC using muscimol/baclofen infusions impairs sequencing. This effect was shown to be selective to performance of sequences that varied spatially from trial to trial; thus, no effects of vlPFC inactivation were observed for performance of a fixed response sequence. Once animals could learn a heuristical strategy for a self-ordered fixed sequence, vlPFC inactivation no longer impaired performance. Chapter 4 investigated the effects of the chemical neuromodulation of vlPFC on self-ordered sequencing using microinfusions of dopamine receptor D_2 antagonist, sulpiride, and $5HT_{2A}$ receptor antagonist, M100907 on performance of variable sequences. These drugs produced contrasting, dose-dependent impairments. M100907 impaired accuracy, while sulpiride impaired error correction. Chapter 5 studied effects of blocking glutamatergic receptors in a region of the caudate nucleus to which the vlPFC projects, but no significant effects on sequencing accuracy were observed, although there were large effects on perseverative errors in 2 out of 3 animals.

The findings are discussed in Chapter 6 in terms of the functioning of the vlPFC and its possible role in controlling flexible response sequencing and working memory. The findings are shown to be of relevance for psychiatric disorders such as obsessive compulsive disorder (OCD) and schizophrenia, which show functional dysconnectivity of the vlPFC in association with response sequencing impairments.

Abbreviations

5,7-DHT - 5,7-dihydroxytryptamine

6-OHDA - 6-hydroxydopamine

5HT - 5-hydroxytryptamine (serotonin)

$5HT_{(X)}$ - 5-hydroxytryptamine receptor x

ACC - Anterior cingulate cortex

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA - Analysis of variance

AP - Anterior-posterior

ATD - Acute tryptophan depletion

CANTAB - Cambridge neuropsychological test automated battery

CNQX - Cyanquixaline

CS+ - Conditioned stimulus

$D_{(x)}$ - Dopamine receptor x

dlPFC - Dorsolateral prefrontal cortex

DLS - Dorsolateral striatum

DMS - Dorsomedial striatum

dmPFC - Dorsomedial prefrontal cortex

DREADD - Designer receptors exclusively activated by designer drugs

FSI - Fast-spiking interneuron

GABA - Gamma aminobutyric acid

$GABA_A$ - GABA receptor A

$GABA_B$ - Gaba receptor B

IR - Infrared

ITI - Inter-trial interval

LM - Latero-medial

lPFC - Lateral prefrontal cortex

LSD - Lysergic acid diethylamide

mPFC - Medial prefrontal cortex

MRI - Magnetic resonance imaging

MSN - Medium spiny neuron

Musbac - Muscimol/Baclofen

NACWO - Named animal care and welfare officer

NMDA - N-Nitrosodimethylamine

OCD - Obsessive compulsive disorder

OFC - Orbitofrontal cortex

OTS - One touch stockings of Cambridge

PBS - Phosphate buffered saline

PFC - Prefrontal cortex

R - Receptor

SEM - Standard error of the mean

SMA - Supplementary motor area

SNT - Sequential nosepoke task

SOC - Stockings of Cambridge

SSRI - Selective serotonin reuptake inhibitor

SWM - Spatial working memory

VT - Vanishing time

vlPFC - Ventrolateral pre-frontal cortex

WCST - Wisconsin card sorting test

Table of contents

1. General Introduction

The set of experiments that make up this thesis investigated the causal role of the ventrolateral prefrontal cortex (vlPFC), a region of the frontal lobes, in performance of self-ordered spatial response sequencing. In this Introduction, I will provide an overall perspective on the structure, connections and functions of the frontal lobes, particularly regarding their role in control of so-called executive functioning, with a more in-depth focus on the lateral prefrontal cortex. This will be followed by a review of how this structure mediates goal-directed behavioural sequences, under a range of conditions, from the perspectives of learning theory and underlying neural circuitry.

1.1. Frontal lobes

The frontal lobes are the largest of the four lobes in the human brain, illustrated in **Fig. 1.1**. As its name suggests, it is the most anterior of the four lobes. It is positioned anterior of the parietal lobe, in front of the central sulcus, and above and in front of the temporal lobe. The frontal lobe can roughly be divided into motor regions and the prefrontal cortex (PFC). The prefrontal cortex refers to that part of the frontal lobe anterior of the motor cortices.

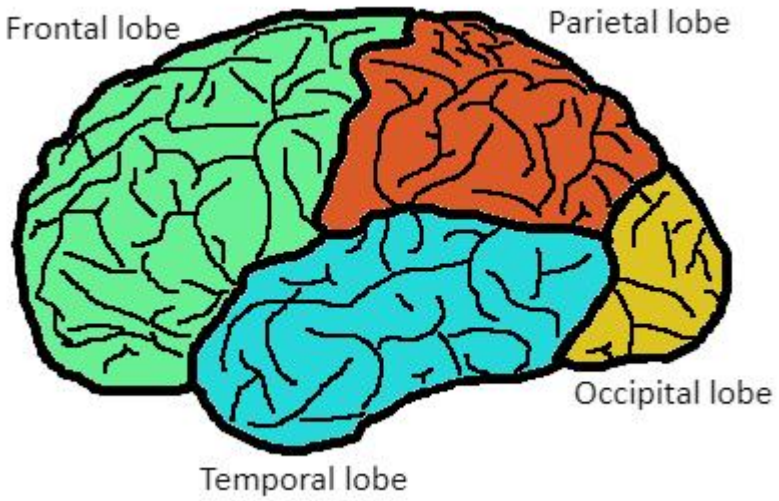


Figure 1.1 The four lobes of the human brain.
Image illustrate the frontal lobe (green), parietal lobe (orange), temporal lobe (blue) and occipital lobe (yellow).

In our drive to understand how the brain controls behaviour, the PFC is perhaps the most intriguing area to study. The attributes that make us, "us", are generally attributed to the PFC. This insight originally came from a work-related accident, where the railroad foreman Phineas Gage, had a 1.1m long tampering rod driven straight through his skull, damaging his PFC (Harlow, 1848). Phineas made a miraculous recovery but suffered from personality changes so severe his friends said he was 'no longer Gage' (Harlow, 1868). The PFC is held as that part of the brain most relevant for executive functions and disturbances in these processes, such as for Phineas Gage, are severely detrimental. Executive functions are mental processes / cognitive functions that control thought and action, which facilitate goal-directed behaviour. Generally, the processes contained within executive functioning are, working memory, inhibitory control and cognitive flexibility (Friedman et al., 2006). Working memory refers to the ability to update, delete and manipulate information in short-term memory and is also the executive function that correlates highly with intelligence, as measured by IQ (Friedman et al., 2006). Inhibitory control refers to the processes that prevent the initiation of behaviour that is inappropriate in the current situation or context. Cognitive flexibility refers to the ability to change behaviour in accordance with environmental changes, to maximise a desired outcome. If more than one executive function is required for a behaviour it is referred to as higher-order executive functioning and the concept of planning response sequences for goal-directed behaviour is an important part of this. Studying human patients with lesions of the frontal lobe have provided important, clinically relevant, information for neuroscience and psychology. Three key studies that have provided insight into the involvement of the frontal lobes in working memory, cognitive flexibility and inhibitory control are presented below.

1.1.1. Human frontal lobe lesions and executive function

Owen et al (1990) investigated the performance of patients with lesions of the frontal lobe on three different computerised cognitive tasks. One task aimed to investigate spatial short term memory, the second task aimed to investigate spatial working memory (SWM) and a third task assessed higher level planning. The SWM task and the planning task will be referred to on several occasions in this thesis and are described in detail in **Box 1.1**. Patients with frontal lesions showed no impairment in performing an instructed sequence of visuo-spatial

responses, indicating that performance of simple sequences from short term-memory is still intact in patients with frontal lesions. However, when the prefrontal lesion group had to perform the SWM task, which required them to search by themselves through an array of boxes to find hidden tokens, they made more within- and between-search error; the former referring to revisiting a box found to be empty on that trial and the latter referring to revisiting a box already found to contain a token on a previous trial. Furthermore, they were less able to organise their behaviour in a strategic way, which would optimise performance by reducing the working memory load of the task. On the planning task, patients with frontal lesions had prolonged thinking times, required significantly more moves to complete a trial and hence had fewer solutions in a minimum number of moves. Collectively, this indicates that patients with frontal lesions can successfully generate visuo-spatial sequences from short term memory but when the sequence stored in short term memory needs to be manipulated, as in the SWM task, patients revisit previous responses and are also impaired at successfully organising their behaviour in an optimal way. This was further emphasised in the higher-order planning task where patients with prefrontal lesions made more errors, performed fewer optimal solutions and took longer when they were required to generate and execute a sequence to solve the task.

Impairments in cognitive flexibility have also been found in patients with lesions of the frontal lobe (Milner, 1963). Patients were tested on the Wisconsin Card Sorting Test (WCST). In the WCST patients are presented with four stimuli cards that contain different shapes, number of shapes and colours. Patients are then presented with a deck of 'sorting' cards, which contain cards of the same dimensions as the stimulus cards and asked to match one sorting card at a time with the stimulus cards. The subjects were not told on what dimension (number, shape or colour) they should sort the cards and were only told if their response was correct or incorrect. Subject were not allowed to correct their response if it was incorrect and were required to choose a new sorting card. Once the patient made ten correct responses in a row, the rule changed without their knowledge and the subjects had to adapt their responding based on the negative feedback received. Patients with lesions of the dorsolateral frontal cortex, as opposed to patients with lesions of anterior parts of the cortex, were severely impaired in this task; they showed a strong tendency to perseverate, i.e. repeat incorrect responses.

Patients with frontal lesions also show inhibitory control impairments as exemplified in a study by Drewe (1975). In that study, patients were tested in a go - no go task, where two

Box 1.1 The Cambridge Neuropsychological Test Automated Battery (CANTAB) SWM and Stockings of Cambridge (SOC) tasks

The CANTAB SWM is a computerised task which assess executive function by performance of sequences of visuo-spatial moves. In this task subjects are presented with a number of identical stimuli on a touch screen. With increasing number of stimuli, the task become increasingly difficult. Subjects need, through a process of elimination, find a token hidden behind a stimulus. The goal is to collect as many tokens, as the number of stimuli presented, but only one token is presented per trial. Once a token has been found, the trial restarts and the token is placed under a new stimulus. The token will never return to a spatial location where it has already been presented. Subjects continue performing new trials until they have found a token for each individual location. Errors are made by searching a spatial location where a token has already been found, called between-search errors or searching tokens which they already know to be empty on that trial, called within-search errors. The task also provides a measure of strategy. For optimal performance, subjects should organise their behaviour to perform a predetermined sequence until they found a token (Owen et al., 1990). Once a token is found, they should simply restart the predetermined sequence, but omit the responses where a token was already found.

The CANTAB SOC is a computerised test of higher-order planning based on the Tower of London task, introduced by Shallice (1982). Subjects need to use problem solving skills to match two stimuli presented on the screen concurrently. Each stimulus depicts three stockings containing three balls (not unlike snooker balls in a pocket), to solve the task successfully subjects need to move the balls within one stimulus (bottom) to match the other (top). The sockets can hold a different number of balls and only the ball on top can be moved. When a ball is placed in a socket it will fall to the bottom on that socket. One ball is moved at the time and participants are instructed to make as few moves as possible. Data are collected on the difficulty level reached, number of moves performed, the number of perfect solution responses as well as response and thinking latencies.

separate cues indicated whether a subject needed to make a response or inhibit that same response. For successful performance subjects were required to make a quick response, when indicated by the go-cue, and successfully inhibit a response when presented with the no-go cue. Patients with frontal lesions took longer to reach learning criterion, with fewer patients passing criterion and more errors being made over the entire task. Both control groups and patients with frontal lesions showed a preference towards 'go' responses, but patients with lesions made more 'go' responses. Indicating a deficit in inhibiting responses towards a non-preferred response.

These three studies demonstrate that an intact frontal lobe is important for all aspects of executive functioning and that damage to the area leads to cognitive deficits in a variety of tasks.

1.1.2. Prefrontal cortex

The three tasks presented above provide a brief overview of frontal lobe regulation of executive functioning. For complex creatures like humans, control of cognition by well-developed frontal lobes is fundamental to behaviour that can adapt and react to the environment. This control is essential for complex behaviours such as language production (Alexander et al., 1989), social cognition (Farrant et al., 2005) and regulation of emotion (Roberts, 2020). When discussing the frontal lobes, a useful distinction can be drawn between the regulation of "hot" and "cold" cognition, both dependent on frontal lobe functioning. "Cold" cognition, the subject of this thesis, refers to cognition independent of emotional influence, examples of this are the cognitive tasks already presented in this chapter, which strictly measures these functions; while "hot" cognition, is cognition with emotional influence (Roiser and Sahakian, 2013). In tasks designed to asses "hot" cognition, executive functioning is still assessed, but choices in the task can affect winning or losing currency, amount of reward or lead to aversive outcomes.

The frontal lobe is not a unitary structure and it can be divided into several areas which serve different functions to regulate behaviour. Loosely speaking, these areas can be divided into motor areas, the lateral PFC (lPFC), orbitofrontal cortex (OFC) and medial PFC (mPFC). Non-human primates have been essential in our understanding of each of these different areas. Both the rhesus macaque (Petrides et al., 2012) and common marmoset (Burman et al., 2006;

Burman and Rosa, 2009) have well-defined parcellations of the prefrontal cortex. However, some differences between species is noticeable already at the brain surface level, marmosets, unlike humans and rhesus macaque have a smooth brain surface. Nonetheless, a degree of homology exists between human and primate PFC, particularly with regards to cytoarchitecture and connectivity. Although, even if the PFC can be divided into areas of similar cytoarchitecture in both humans and non-human primates, there are differences within these. In certain areas of human PFC, it is possible to find separate fields, such as changes along a rostro-caudal axis. These more subtle changes are sometimes not reflected across species. One example of this is area 12/47l, which in humans can be divided into a rostral and a caudal area. However, in the marmoset brain only a trend can be seen between rostral and caudal parts of area 12/47l, no clear boundaries can be drawn (Burman and Rosa, 2009). Differences aside, the homology between species make non-human primates a useful tool in investigating the function of the PFC.

A very brief description will be presented below of two areas mentioned later in the introduction, motor areas and the OFC, followed by an in-depth review of the lPFC.

1.1.2.1. Motor areas

The motor areas of the frontal lobe are located anterior to the central sulcus and separate the frontal lobe from the parietal lobe. The motor cortex can be divided into three different areas, the primary motor cortex, the premotor cortex and the supplementary motor area (SMA). All of these areas act to facilitate cognitive control of motor behaviour. The primary motor cortex acts to facilitate the execution of movement, while the supplementary motor area acts to plan sequences of movement (Roland et al., 1980). The premotor area is attributed to be involved in sensory-integration, connecting sensory events with voluntary movements (Mitz et al., 1991).

1.1.2.2. Orbitofrontal cortex

The OFC is the area of the frontal lobe located behind the orbits of the skull; it consists of several areas which all act to assign and update value and is thus important for decision making. The OFC is involved in associating expected reward value with stimuli to guide goal-directed behaviour. Marmosets with lesions of the OFC are able to successfully perform extra-dimensional (higher-order) shifts in a task analogous to WCST, but show an impairment in

performing shifts, specifically reversals, within the dimension, as they are unable to update the reward value of the stimuli (Dias et al., 1996). A study in rhesus macaques showed separable roles for the anterior and posterior OFC in updating of values and goal representation (Murray et al., 2015). They showed that inactivation of the posterior OFC impaired the ability to update the current value of expected reward, while inactivation of anterior OFC impaired the ability to convert these values into appropriate behavioural goals. OFC also plays an important role in the regulation of emotion (Agustín-Pavón et al., 2012; Izquierdo et al., 2005). This regulation of "hot" cognition is however outside of the scope of this thesis.

1.2. Lateral PFC

lPFC is an area of the PFC which is highly involved in executive functioning and of particular relevance on account of its role in higher-order planning. The lPFC can be divided into two areas, the dorsolateral PFC (dlPFC) and vlPFC. The focus on this introduction will primarily be on vlPFC, but dlPFC will also be considered.

1.2.1. Defining the lPFC

Our knowledge of the lPFC comes almost exclusively from findings in human and non-human primates, due to the apparent lack of an homologous or analogous area in rodents (Preuss, 1995). **Fig. 1.2** illustrate that in both human and non-human primates, the lPFC can be divided into vlPFC and dlPFC, which can be further divided into separate Brodmann areas. In this thesis, the vlPFC is defined as Brodmann area 47/12, but vlPFC can also include areas 45 and 44. Area 47 in humans is rostral and ventral of area 45, and corresponding to the pars orbitalis of the inferior frontal gyrus (Brodmann, 1909). A similar area in primates, part of the inferior convexity, was first labelled as area 12 (Walker, 1940) and the corresponding area in rhesus is now defined as the area rostral and ventral to area 45, continuing ventrally as far as the lateral orbital sulcus and labelled 47/12 (Petrides and Pandya, 2002). The corresponding area in the common marmoset was previously referred to as 45/12, but has been parcellated into two distinct areas where area 47/12 lies rostral and ventral of area 45 (Burman and Rosa, 2009). dlPFC refers to area 46 and/or 9/46 for humans and macaque, as defined in (Petrides and Pandya, 1999) and area 46 in the marmoset, as defined in Burman et al (2006).

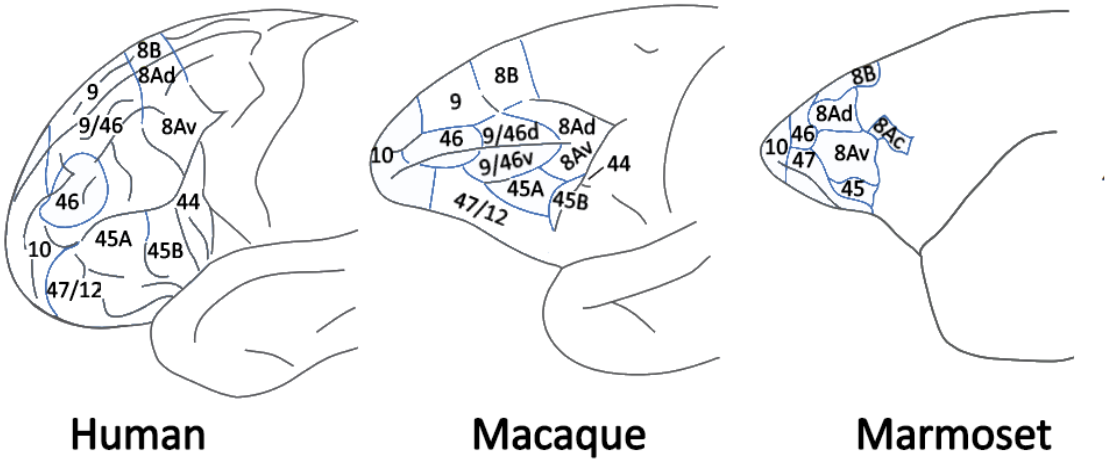


Figure 1.2 Lateral surface view of PFC in human, macaque and marmoset. Areas are labelled according to Petrides et al (2012) for human and macaque, and Paxinos et al. (2012) for marmoset. Image adapted from Roberts (2020).

1.2.2. vlPFC Connectivity

Before reviewing the functional role of vlPFC, it is useful to review the connectivity of the vlPFC. Roberts et al (2007) investigated the connectivity of marmoset PFC using anterograde and retrograde tracers and findings from their study are presented below.

From the injection sites (orbital, medial, dorsal, lateral (the vlPFC injection site)), lateral PFC had the highest connectivity within the PFC and also the most widespread connections outside of the frontal lobes. It received dense bilateral projections from area 8 and premotor area 6. It also received projections from rostral dorsolateral regions 46, 9 and 10. Rostral and caudal regions of OFC (including agranular insula and olfactory cortex) also projected to vlPFC. All the intra-PFC connections were reciprocal. No connections were observed with the rostral and ventral regions on the medial surface apart from a few in the caudal aspect of area 32. This is broadly consistent with the connectivity of area 12 in macaque, which have more orbital connections than medial, with exception for 12o that have connectivity with both networks (Petrides and Pandya, 2002).

The lateral injection site also had the most extensive connections outside of the frontal lobe, also consistent with findings in the macaque. vlPFC had significant connections with sensory association areas. It connects with visual association areas of the inferotemporal cortex, the

polysensory region in and around the superior temporal sulcus and auditory secondary and association regions. The vlPFC also has reciprocal connections with extrastriatal regions including middle temporal visual area, middle temporal crescent visual area, a dorsal visual area and ventral visual areas. The lateral PFC projects to the dorsal head of the caudate nucleus, an area in between the dorsal projections dorsolaterally and the medial/orbital projections medially, occupying the central position, much in line with macaque projections. Similar to the other injection site investigated, the projections run longitudinally along the full length of the head and body of the caudate nucleus, as well as entering the tail.

There was also extensive neuronal and terminal labelling in posterior parietal cortex, area 7. No direct connections with the hippocampus were observed, but it receives connections from the posterior parahippocampal cortex, the projections back from lateral PFC to the parahippocampal cortex are however scarce. The lateral PFC injection site also has reciprocal connections to the amygdala, in contrast with the dorsal injection site.

1.2.3. Functional specialisation within the lPFC

The functional specialisation within the dorsal and ventral lPFC have been the subject of many publications. Many theories were originally based on a proposed role, for both areas, in working memory. This arose from early findings that lesions of the frontal association areas impair a spatial delayed response task (Jacobsen, 1936), in which animals were shown food being hidden under one of two identical objects, but needed to wait until they make a response. Much later, with more sophisticated techniques, it was shown that cells in the PFC show delayed-related activity (Kojima and Goldman-Rakic, 1982). Goldman-Rakic (1988) proposed, based on contrasting anatomical connectivity that the role of dlPFC and vlPFC in working memory differed primarily with regards to the nature of the information processed. They suggested the primary role of dlPFC was to process spatial information (“where”) while vlPFC processed features (“what”). This theory has been proven incorrect, or at least over simplified; neural recordings show that delay-activity for 'what' and separately 'where' can be found in both parts of the lPFC, along with neurons that show activity for both 'what' and 'where' (Rao et al., 1997). A separate influential theory suggested that the regions differ in how they process information, rather than what they process (Petrides et al., 1996). Petrides

et al suggested that dlPFC monitors and manipulates information from short term memory and that vlPFC encodes and retrieves information held in association areas.

However, even though vlPFC contains neurons which exhibit delay-dependent activity, this region is not necessarily required for maintaining information during a delay. Rhesus macaques with ablative lesions of the vlPFC are impaired at performing a match-to-sample task, even without a delay. However, once retrained they are able to successfully complete the task with a delay of up to 8 seconds (Rushworth et al., 1997), indicative of a role for vlPFC in action selection and attentional control, rather than working memory. Lesions of the vlPFC also impair higher-order shifting (Dias et al., 1996), but leave simple reversal of reward-associations intact. However, if novel stimuli are introduced once animals have been trained on a reversal learning 'set', vlPFC is required for successful reversal (Rygula et al., 2010). Collectively, this indicates a role in attentional selection and associating stimuli with rules for responding. Learning of these rules are dependent on PFC but ultimately serve to reduce prefrontal task dependence on complex cognitive tasks, as they potentially reduce the working memory requirements by facilitating habits. Indeed, ablation lesions of the vlPFC impair implementation of a reward-maximising strategy (Baxter et al., 2009) as well as diminish a successful 'win-stay' and 'lose-shift' strategy on a visuomotor learning task (Bussey et al., 2001). These tasks all require visual discrimination, but evidence for a role in organising behaviour extends beyond tasks that require visual discrimination. vlPFC excitotoxic lesions impair performance of a spatial self-ordered sequencing task, suggesting a role in organising self-ordered responses even when visual stimuli are identical and no feature discrimination was required (Walker et al., 2009a).

Efforts have been made to integrate the vast amounts of information available across different executive functions (Sakagami and Pan, 2007; Tanji and Hoshi, 2008). These efforts suggest a role for lPFC in integration of sensory and motivational information to compute plans that act to chain together separate voluntary actions to achieve a desired outcome.

Supported by an extensive literature review, Tanji and Hoshi (2008) suggested a role for vlPFC in behavioural planning through first-order executive processing by active retrieval and selection of information. They suggest that dlPFC is involved in behavioural planning of higher-order behaviour through manipulation, monitoring and integration of information.

Sakagami and Pan (2007) focused primarily on the vlPFC and suggested that vlPFC, through its connection with subcortical areas and orbitofrontal cortex, integrates sensory and motivational information to compute adaptive codes based on behavioural significance. These codes lead to deliberate decision making and are key to goal-directed behaviour. They suggest that the codes contain sensory and motor information and that they can either directly affect motor output through connections with motor areas or indirectly through integration with dlPFC.

From these findings it can be said that these areas serve different, but often complimentary functions; likely explained by a difference in how they process information, as opposed to what information they process, as suggested by Petrides (1996).

1.2.4. Establishing a role for vlPFC in the organization of behaviour

Performance of the delayed alternation task is impaired following lesions to the inferior convexity (Iversen and Mishkin, 1970). In this task, subjects are required to alternate their responding between two choices, left or right, displacing a plaque to access a food reward. The responses are temporally associated, as the correct response alternates for each trial. For successful performance, subjects are required to have a simple plan; on a given trial, make the response that was not rewarded last trial. This requires several aspects of executive functioning. Animals need to remember where reward was located on the last trial, inhibit the incorrect response and flexibly update this information for each trial. The potential plan used in the delayed alternation task is simple, but is available to guide responding. However, there are tasks in which animals are required to have a more complex plan to guide behaviour. In sequencing tasks, where animals are required to perform a chain of responses to reach an ultimate goal, they need to structure their responding for optimal performance. This is particularly true when responding is self-ordered and subjects cannot depend on external cues to guide their performance. One such task is the self-ordered sequencing task, adapted from Collin et al (1982) and used by Passingham (1985) to asses effects of prefrontal lesions on working memory performance. In this task, monkeys were presented with 25 holes in a 5x5 grid, each hole covered by a door. For each trial, a peanut was hidden behind a door and animals were required to search through the grid to find a peanut. For optimal performance, subjects had to organise their behaviour as they search through this grid. Rhesus monkeys

seemed to organise their responding, but in a rather arbitrary fashion, and no clear pattern could be observed. However, performing sequences of up to 25 responses makes the task very dependent on working memory. Studies using shorter sequences have demonstrated that, over-time, rhesus macaques performing a self-ordered sequencing task develop a distance minimising strategy (Taffe and Taffe, 2011), not dissimilar to solutions to the traveling salesman problem (Lawler, 1985; Menzel, 1973). Briefly, the travelling salesman problem is an optimisation problem which asks, what is the shortest route to visit a given number of locations, without revisiting previous locations. On account of the diversity of tasks that require an intact vlPFC for optimal organisation of behaviour, it can be suggested that vlPFC is required for learning or executing behavioural plans or strategies. The causal role of vlPFC needs to be studied in tasks that require planning of behaviour, while minimising factors like visual discrimination and working memory. Self-ordered response sequencing tasks can be designed to isolate these processes.

1.3. Sequential behaviour

The importance of studying order in thought and action to understand cerebral function is not novel, nor is it simple. Psychologists in the early 20th century suggested that a sequence, no matter what sequence of responses, consists of individual stimulus-response association that are linked to each other by training (Pavlov, 1927; Skinner, 1934). Lashley (1953) outlined the problems faced by chain theory in explaining serial order and acknowledged its complexity:

"The problems of the syntax of action are far removed from anything which we can study by direct physiological methods today, yet in attempting to formulate a physiology of the cerebral cortex we cannot ignore them. Serial order is typical of the problems raised by cerebral activity; few, if any , of the problems are simpler or promise easier solution. We can, perhaps, postpone that fatal day when we must face them, by saying that they are too complex for present analysis, but there is danger here of constructing a false picture of those processes that we believe to be simpler. l am coming more and more to the conviction that the rudiments of every human behavioral mechanism will be found far down in the evolutionary scale and also represented even in primate activities of the nervous system."

Lashley referred to several processes in human cognition, many from verbal language, which could not be satisfactorily explained by chain theory. His arguments had less of an immediate effect on animal cognition, but Lashley seemed to have been right in his conviction, as there is now enough evidence to persuasively argue that the same critique of chain theory also applies to animal cognition (Terrace, 2005).

1.3.1. What are sequences important for?

Previously in this chapter it was suggested that studying sequences is important for understanding how goal-directed actions are planned. The importance of studying sequences in laboratory animals extend beyond goal-directed planning. Maybe the most advanced form of sequencing humans perform is language. In language, sequences of letters form words and sequences of words form sentences. These sentences follow abstract grammatical rules and it has been suggested that there are at least five systems that represent sequence knowledge in human language (Dehaene et al., 2015). Primates can be trained to perform spatial sequences which adhere to abstract grammatical rules and offers a platform to study the neural basis of these rules (Jiang et al., 2018). However, sequences are not always that complex. Important sequences for behaviour, across species, are motor skills. Several movements organised temporally for performance, comprises a sequence of actions. When studying performance of sequences experimentally, the behaviour that needs to be planned often consists of discrete movements; it could be, for example, a marmoset that needs to move its body and arms to make a response (Walker et al., 2009a) or it could be a rhesus macaque making self-ordered saccades (Hasegawa et al., 2004). However, for the scope of this thesis, the interest is not in motor action per se, it is in the cognitive organization of these response events. Studies investigating learning of motor skills will however be reviewed as they provide important insight into the wider neural network involved in performance of sequences.

1.3.2. Rodent studies on performance of response sequences: a focus on the striatum

1.3.2.1. Behavioural control of sequences

Studies on performance of response sequences in rats and mice have generated important knowledge on how such sequences are performed. In particular, rodent studies have been

important for understanding the relationship between goal-directed control and habitual/automatic performance of sequences, especially in relation to the basal ganglia.

In simple instrumental conditioning, where an animal needs to make a response, such as lever press, for a reward, training is an important factor for how behaviour is controlled. During acquisition, behaviour is controlled by an association between the action and the outcome, if the reward is devalued, animals stop responding (Adams and Dickinson, 1981). With training, behaviour shifts from goal-directed to habitual, meaning that behaviour is under control by stimulus-response processes and animals are no longer sensitive to changes in reward value (Balleine and Dickinson, 1998). However, what happens when animals, instead of making a single response for outcome, are required to perform a chain of heterogenous responses?

In a sequence, the different responses in that sequence occur more proximally and distally in relation to reward, and these responses could theoretically be under different forms of behavioural control. Balleine et al (1995) trained rats to perform a sequence of two responses and tested subjects in extinction, in either a food deprived or undeprived condition. The primary sequence used required animals to perform a lever press and a chain pull, in a pre-determined sequence. A further sequence was introduced which consisted of a lever press and magazine panel push to access reward. These two tasks were used to assess the effect of motivational control over different responses in a sequence. The main finding was that control of behaviour varies across the chain of responses in relation to reward proximity. Their findings indicate that responses distal to reward are under goal-directed control while responses proximal to reward are more driven by stimulus-response associations. The same lever press, chain pull sequencing task has also been tested under outcome devaluation, and indeed a similar conclusion was found (Balleine and Dickinson, 2005) such that a response temporally near to the reward was insensitive to devaluation while the response further away was sensitive. Studies have been performed in rodents to investigate if control of behaviour in a sequencing task differs between rats that are moderately or extensively trained (Garr and Delamater, 2019). Subjects were trained to perform a left lever response, followed by a right lever response, for a sucrose pellet. No other sequences were rewarded. The extensively trained group (60 days) showed a higher degree of sequence automaticity (measured as reduced latencies and a more homogenous pattern of responding) as compared to the moderately trained group (20 days). After training, subjects were tested during extinction,

with reward devalued. They found that both groups showed goal-directed control of behaviour. However, the goal-directed control of behaviour was manifested in different parts of the sequence. The moderately trained group showed goal-directed control of initiation, but contradictory to previous findings, the extensively trained group showed goal-directed control of termination. Two important conclusions from the study are that highly automatized sequences are not by definition habitual and that with extensive training, goal-directed control can occur across a chain of responses.

In the second paragraph of this section, the concept of habitual control of instrumental behaviour was defined as the property of being insensitive to outcome. However, Garr and Delamater (2019) demonstrated that even though an extensively trained group showed high level of automaticity, the behaviour was still sensitive to outcome. These findings demonstrate that not all automatized behaviours are habits. These behaviours are referred to as skills (see Robbins and Costa (2017)).

It has been suggested that, if a sequence contains more responses than an initiating and a terminating response, goal-directed control of these intermediate actions would decrease with reward proximity (Keeler et al., 2014). A possible critique of the sequencing studies presented above is the short sequence of only two responses, in performance of two response sequences, one response initiates the sequence and the next terminates the sequence and thus, it is not possible to study responses that occur-between the two. Nonetheless, it can be concluded that goal-directed control of behaviour varies across a chain of responses and that training can move the element that is under goal-directed control within this chain.

1.3.2.2. Striatum involvement in learning and performance of sequences

Tasks presented below investigate the role of the striatum on performance of sequences by modulation of the striatal direct and indirect pathways. These pathways act to coordinate movement and are modulated by the neurotransmitter dopamine. The direct pathway is primarily modulated by dopamine receptor 1 (D_1) while the indirect pathway is primarily modulated by dopamine receptor 2 (D_2) (See e.g., Gerfen and Surmeier, 2011).

Keeler et al (2014) designed two sequential nosepoke tasks (SNT) with longer chains of responses. Rats were trained to perform either a sequence of 7 or 4 responses in a nine-hole

box. In the 7 response SNT, animals performed a sequence of spatially separated responses (numbered 1-5 from left to right), in the order 3 -> 1 -> 2 -> 3 -> 4 -> 5 -> reward, in the 4 response SNT, animals only performed responses 3 -> 4 -> 5 -> reward. Performance of the two tasks were investigated under systemic treatment with different dopamine agonists and antagonists. In a series of experiments on both the 4 and 7 task they showed that D_1 signalling was important for early parts of sequence performance, while D_2 signalling was important for termination of the sequence. They argued (supported by a literature review) that basal ganglia D_1 acts to invigorate initiation by preparing the response, while basal ganglia D_2 acts to shape and select responses within the prepared set. This task design is much more promising due to how sequence length can be altered with only minimal change in motor requirements, thus isolating effects of sequence related processing.

Garr and Delamater (2020) followed up their original study on performance of sequences with a chemogenetic technique, designer receptors exclusively activated by designer drugs (DREADD) (Roth, 2016). Inhibitory receptors that could be selectively activated were expressed in cells containing either D_1 or D_2 receptors in either the dorsolateral striatum (DLS) or the dorsomedial striatum (DMS). Garr and Delamater investigated these receptors in the different areas on learning and/or execution of a sequence. They found that a neural circuit including the indirect pathway originating from the DLS (homologous to putamen) was involved in goal-directed reinforcement of the sequence. The DMS (homologous to caudate nucleus) was involved in both goal-directed initiation and completion of the response sequence. The indirect pathway was suggested to be responsible for sequence initiation while the direct pathway was responsible for completion. These findings are contradictory to previously presented findings by Keeler et al (2014), but it is difficult to compare studies due to differences in the task and neurobiological techniques used to manipulate dopamine. Balance between indirect and direct pathways in the dorsal striatum has been suggested to be shaped by cortical motor areas. For example, Rothwell et al (2015) showed that for serial-order performance, activity in the indirect and direct pathways is shaped by signals originating from the secondary motor cortex that terminate on medium spiny neurons (MSN) of the direct pathway.

1.3.2.3. Motor skills and striatal dopamine

If we consider the sequencing tasks above, animals, with training, are essentially developing a motor skill. So, insights from motor skill learning can provide useful knowledge on sequence learning. Learning and performance of a motor skill task, rotarod, does also require interplay between DLS and DMS (Yin et al., 2009). Those authors showed that the DMS is important for early stages of training, but the DLS is more important for later stages of performance and that learning of a skill leads to regional changes in synaptic transmission. They also showed that D_1 or D_2 antagonists impaired early performance of the task, while after extensive training only the D_2 antagonist impaired performance - suggestive of a role for both the indirect and direct pathway in learning of a motor skill, while the indirect pathway is also important for performance of that learnt skill.

A lot of work with very sophisticated techniques have been performed on sequence learning and performance using the fixed ratio task in which rats respond repetitively to obtain reward, e.g. every x number of responses. Such sequences are limited however as they only contain a homogenous chain of responses. Some relevant findings will nevertheless be considered briefly.

Cells in the nigrostriatal circuit show selectivity for the 'start' and 'stop' in a sequence and depletion of NDMA-receptors in the striatum impair sequence learning by affecting phasic firing of dopamine neurons and plasticity of MSNs (Jin and Costa, 2010). In contrast to previous findings by Garr and Delamater (2020), it has also been reported that both the indirect and direct pathways are involved in action initiation, suggesting that the direct pathway promotes the use of a specific motor program while the indirect pathway inhibits undesired motor programmes (Cui et al., 2013). Further studies showed that both the indirect and direct pathway were active during initiation, but exhibited differential activity during performance (Jin et al., 2014). These circuits were then manipulated during performance and it was demonstrated that they need to act collaboratively to support successful initiation and execution of a sequence (Tecuapetla et al., 2016).

1.3.2.4. Rodent prefrontal cortex on performance of sequences

Few studies in rodents have investigated prefrontal cortical manipulations on response sequences, but a role has been established for dorsomedial frontal cortex in organising action

sequencing (Bailey and Mair, 2007). Ostlund et al (2009) investigated performance of a lever-based response sequencing task in rats after lesions to dorsomedial PFC (dmPFC). Two out of four sequences were rewarded, Left-Right and Right-Left, each giving a unique type of reward. Lesioned animals were able to learn the task but were dependent on a different mechanism for performance. Lesions of the dmPFC left sensitivity to outcome-devaluation intact, but they only withheld the terminal response of the devalued sequence; in contrast to sham animals, which successfully withheld any responding when reward was devalued. Indicating that the lesioned group were unable able to group the two separate responses together, by formation of an action chunk.

Rat dmPFC has been suggested to share functional and anatomical properties with SMA in primates and humans (Donoghue and Wise, 1982; Passingham et al., 1988), indicating a potential role of prefrontal motor areas in sequence processing.

1.3.3. Primate studies on performance of response sequences: a focus on fronto-striatal loops

Sequential performance in primates have been studied extensively and provided important information on the learning and execution of response sequences. Important findings will be reviewed, with a focus on lPFC.

An influential theory based on findings in primates, proposes that there are two parallel networks for learning sequential procedures (Hikosaka et al., 1999). The findings are based on how primates learn sequential procedures in conjunction with cell recordings and acute manipulations of specific brain areas. They used a '2 x 5' task, in which subjects need to, in a fixed order, perform five sequences of two responses, called a ‘hyper-set’. Responses were made on a keypad with 4 buttons. It is indicated to subjects by flashing lights which two responses they need to make, but not in which order, and they are required to perform the correct sequence through trial and error. Once a sequence of two is completed, they move on to the next sequence of two, until they have performed the entire hyperset. If an error is made, the subjects are required to restart from the first sequence, meaning that they learn the entire hyperset as a unique sequence. During performance of these trials there is a distinction between “early” learning stages and “late” performance stages (Rand et al., 1998). In the early stages of performance, the sequences are not specific to hand and sequence

order, the sequences are performed slowly, without any anticipatory movement. In the later stages, performance is dependent on what hand performs the task and in what order the component sequences are made. The sequence is also performed in a rapid manner with anticipatory movements (Miyachi et al., 1997). Sequence performance remains stable over extended periods without practice, as opposed to early stages where performance diminished quickly (Hikosaka et al., 1995). Hikosaka et al (1999) argue that there are two parallel networks that independently learn a sequence. During early learning these systems work hierarchically, one feeding information to the other to guide actions. During repeated performance these systems start working in parallel to learn the sequence. The first network is based on spatial sequences (visual coordinates) and the second one is based on motor sequences (motor coordinates). The spatial sequence is learnt in anterior basal ganglia and PFC, in loops that quickly learn sequences by modulating attention and working memory. The second system consists of loops between pre-motor cortex and the middle part of the basal ganglia (particularly the putamen); this system acquires the response sequence slowly and cumulatively with long-term practice.

In support of this, the authors present several studies that have examined the roles of the medial frontal cortex, striatum and cerebellar nuclei in performance on these tasks. Single cell recording studies show that several neurons become active when learning a new sequence; these neurons are more predominant in the pre-SMA than in the SMA (Nakamura et al., 1998). Temporary unilateral inactivation of the pre-SMA and the SMA increased sequence errors for new sequences, but not for learned sequences, the effect being stronger after inactivation of the pre-SMA (Nakamura et al., 1999). In the striatum, the anterior part demonstrates a stronger tendency to be activated by new sequences, whereas the middle putamen tended to be more active for learned sequences (Miyachi et al., 2002). This finding is consistent with experiments showing that inactivation of the anterior striatum impairs new sequences, but learned sequences to a lesser degree, and that inactivation of middle putamen impaired learned sequences, but not new sequences (Miyachi et al., 1997). A role for cerebellum has also been established in performance of “late” sequences. When the dorsal or central part of the dentate nucleus of the cerebellum was inactivated the number of errors for learned sequences increased (for the hand ipsilateral to injection), whereas performance of new sequences was unaffected (Lu et al., 1998).

The parallel neural network theory remains relevant and explains learning of sequences that needs to be explored "cognitively" through trial and error and subsequentially reduced to motor skills. However, not all cognitive tasks can so easily be translated into a motor skill, some require constant updating of behavioural plans and subsequent translation into motor actions for optimal performance.

Complex networks of brain areas are involved in arranging behaviour into sequences, reflected by the number of brain areas which contain neurons that show selectivity for serial order. This activity reflects different levels of processing of serial-order across domains. These cells can be found in, among other areas, primary motor cortex (Carpenter, 1999), SMA and pre-SMA (Clower and Alexander, 1998), anterior cingulate cortex (ACC) (Procyk et al., 2000), lPFC (Hasegawa et al., 2004), ventral striatum (Shidara et al., 1998), dorsal striatum (Kermadi and Joseph, 1995) and arcuate cortex (Barone and Joseph, 1989). Activity in arcuate cortex is believed to reflect order when oculomotor sequences have to be performed (Barone and Joseph, 1989). The pre-SMA and SMA controls motor components of sequences. Neural activity in both the pre-SMA and SMA reflects individual movement components of the sequence (Clower and Alexander, 1998). They are however separable, SMA contains neurons that reflects the order of the components in relation to each other (Tanji and Shima, 1994) while pre-SMA contains cells that reflects when a motor plan needs to be discarded and replaced by a new motor plan (Isoda and Hikosaka, 2007; Shima et al., 1996). Procyk et al (2000) performed neural recordings of the ACC on a task that required animals to, through trial and error, find the correct sequence of three responses on three buttons. Once three correct responses had been made, animals needed to search again for a new sequence. Neural activity in the ACC changed with different phases of the sequence. Neural activity reflected when animals needed to search for a new sequence, until they had gathered all information, even if they had not yet tested the correct sequence. Once the sequence had been found another group of cells showed sensitivity for repetition. This indicates that the ACC is either involved in reward expectancy or that it acts to monitor the success of the response sequence. Outside of prefrontal cortex, the ventral striatum shows similarities to how the ACC represents sequences and it has been suggested that it helps track progress towards a reward (Shidara et al., 1998). In the dorsal striatum, the caudate nucleus integrates both task and sequence progression information and has been suggested to play a role in

converting temporospatial information to construct, execute and update a spatial plan (Kermadi and Joseph, 1995). The putamen has been reported to be more involved in execution of known sequences (Kimura, 1990). This division between caudate and putamen is generally in line with findings from rodents. However, inactivating both the anterior caudate and putamen impairs performance of "early" sequences in the 2x5 task, while only inactivation of posterior putamen impaired performance of "late" sequences (Miyachi et al., 1997), indicating that there might be a functional difference between anterior and posterior parts of the putamen in primates.

1.3.3.1. lPFC and self-ordered sequencing

The question remains, which area represents higher-order behaviour plans? The lPFC is a likely candidate. An intact lPFC is required for correct performance across cognitive tasks, many of which require higher-order executive functions. The rich connectivity of the lPFC is another compelling argument and the ventral-dorsal gradient in the lPFC allows it to integrate information across domains and functions to form a behavioural plan. There is indeed plenty of evidence in support of this hypothesis.

On performance of a self-ordered sequencing task where animals need to make responses to three unique visual stimuli, a set of neurons in the lPFC reflected task progress, with very little evidence of reflecting working memory load or reward outcome (Hasegawa et al., 2004). lPFC does not only track progress, but reflects both previous and future spatial goals (Genovesio et al., 2006). In a task where subjects in a self-ordered fashion need to move a cursor through a maze to reach a target, cells reflect spatial representations corresponding to immediate and final behaviour goals (Saito et al., 2005). This finding was extended to show that, during a preparatory phase of the task, neuronal activity reflected all three required moves of the cursor. Neuronal activity reflecting the motor action required to move the cursor was not widely found in the lPFC, indicating that it was the movement of the cursor that was planned and not the motor action required to move the cursor (Mushiake et al., 2006). In contrast, arm movements were correlated with activity in the primary motor cortex (Mushiake et al., 2006). As subjects search, through trial and error, for a correct sequence, neural activity initially reflects (un)certainty. During further testing this activity stabilises, indicating perhaps that the lPFC represents subjective knowledge of the action sequence (Averbeck et al., 2006).

Knowledge of sequences can also be represented in categories. Rhesus macaques were trained to perform three different separate movements. These movements were then chained together to compose three different 'categories' or types of four response sequences. The categories were alternating sequences (for example push-pull-push-pull), paired sequences (for example Turn-Turn-Push-Push) or four repeating movements. No visual stimuli represented the categories, subjects were simply 'instructed' to perform a particular sequence based on visual cues. Cell recordings in the lPFC were performed when subjects carried out the task and found to encode the different categories of sequences. This indicates that the lPFC contains self-generated, abstract representations of higher-order categories used for behavioural planning (Shima et al., 2007). The behavioural plan reflected in the neural firing of lPFC was represented before initiation and through decoding analysis prior to initiation, it could predict how the subject would respond (Averbeck and Lee, 2007). This prediction was so accurate that, on erroneous trials, it could successfully predict where in the sequence errors would be performed.

Collectively, this provides strong evidence for a role of lPFC in organising behaviour in higher-order cognitive tasks, especially self-ordered behaviour. The requirement to organise behaviour, particularly self-ordered behaviour, is what drives planning, and planning is ultimately what guides sequences. Planning likely serves to minimise prefrontal-task requirements by allowing for adoption of strategies. There is indeed evidence that search strategies not only improve performance, but also decrease encoding in the lPFC (Chiang and Wallis, 2018). Ultimately, in an unchanging environment where subjects can “exploit” constancy, the plan can be reduced further to a sequence of simple motor actions.

The majority of studies above, investigating lPFC, covered an area both dorsal and ventral of the principal sulcus. In many cases it is unclear whether the area only comprised dlPFC or if vlPFC was also included. Based on the histology presented, area 12 was included in some studies, but it is often difficult to be sure. For this reason, this entire section only refers to the area investigated as lPFC. It is however clear that all studies investigated dlPFC, but not all also included vlPFC. However, overall there is strong evidence that vlPFC plays a key role in performance of spatial self-ordered response sequences (Walker et al., 2009a); this is particularly interesting in the light of vlPFC lesions also abolishing behavioural strategies (Baxter et al., 2009; Bussey et al., 2001).

1.3.4. Human studies on response sequencing

There is support for the parallel neural network theory, described above, in humans (Hikosaka et al., 1998). Also in general agreement with non-human primate findings, the PFC has been suggested to be required for learning motor sequences that are attentionally demanding (Eliassen et al., 2001; Lewis and Miall, 2003). Further to this, similarities can be found in sequence performance after lesions to the basal ganglia in both human patients and primates (Dahms et al., 2020). However, the aim of this section is not to compare the literature for control of response sequences between primates and humans in terms of the entire brain, but to focus on important studies that evaluate the role of human lPFC and also review a potential role for response sequencing impairments in psychiatric disorders.

1.3.5. Human lPFC involvement in performance of response sequences

A key study investigated healthy human subjects on performance of four spatial sequencing tasks and measured brain activity during performance using positron emission tomography (Owen et al., 1996). The first task required subjects to monitor what spatial locations had been illuminated, one at a time, on a blank screen and then, in a self-ordered fashion, select the locations from a grid. The three other sequencing tasks used were; a task identical to the CANTAB SWM (described in **Box 1.1**), a second task which required self-ordered reproduction of a sequence of five responses (highlighted in a grid of 8 locations) and a fixed spatial sequence task, where subjects simply needed to remember a spatial sequence and reproduce it during scanning. The study revealed that both vlPFC and dlPFC increased activity during performance of spatial self-ordered sequences. When subjects simply needed to attend to which stimuli were highlighted and then reproduce these responses in a self-ordered fashion, vlPFC increased activity. vlPFC also showed increased activity for reproduction of a fixed sequence from memory. While only dlPFC showed increased activity during performance of the task which required monitoring of which locations had been presented. On the CANTAB SWM task, in which subjects were required to create and constantly update self-ordered sequencing plans in search for tokens, both areas showed increased activity. The majority of the tasks used required the sequences to be generated internally, but there is also evidence that lPFC plays a role in structuring fixed sequences into action chunks (Bor et al., 2003). These chunks improve performance by reducing cognitive load. In the Bor et al study, healthy human

participants were trained to reproduce sequences presented on a 4 x 4 spatial grid. Some sequences were structured by containing familiar shapes (right angled triangles and parallelograms), allowing the sequence to be performed in an easy recognisable pattern, while some sequences were unstructured (less symmetry and fewer parallel sides). Subjects performed the task in a scanner and functional magnetic resonance imaging (MRI) was used to contrast brain activity between structured and unstructured trials. Increased brain activity was seen for structured trials in the lPFC (area 47, 45 and 44). They suggest that lPFC relates the structured sequence to object-based information (as reflected by increased activity of the fusiform gyrus) and selects an appropriate high-level behavioural plan.

The two studies presented above are correlative in nature, but causal evidence exists as frontal lesions have been demonstrated to impair successful strategy implementation (Owen et al., 1990; Shallice and Burgess, 1991). Considered in conjunction with evidence from non-human primates, these findings cement a role for lPFC in structuring higher-order behaviour plans.

1.3.6. Impaired sequencing in psychiatric disorders

Understanding cognitive deficits in psychiatric disorders present an opportunity to develop new treatment for these disorders. Impairments in behavioural planning and action sequencing are cognitive phenotypes of some psychiatric disorders, reviewed in this segment. Obsessive compulsive disorder (OCD), described in **Box 1.2**, and schizophrenia, described in **Box 1.3**, are the two psychiatric disorders which will be the focus of this section. Impairments in performance of sequences are however not exclusive to these disorders, patients suffering from the neurological disorder Parkinson's Disease also show impairments in sequencing (see e.g., Harrington and Haaland, 1991).

Box 1.2 – Brief Introduction to obsessive compulsive disorder

OCD is a psychiatric disorder characterised by persisting thoughts (obsessions) and/or repetitive behaviour (compulsions). These compulsions and obsessions vary between individuals and include symmetry, washing, checking and forbidden thoughts (American Psychiatric Association, 2013). No matter the symptom group they have a profound impact on the quality of life (Macy et al., 2013). It affects approximately 1-2% of the population during their lifetime (Kessler et al., 2005). First-line treatment for OCD is selective serotonin reuptake inhibitors (SSRI) or the tri-cyclic antidepressant clomipramine (Fineberg et al., 2013). However, a large group of patients are unresponsive and an unmet need for treatment exists (Kellner, 2010). The psychopathology of OCD has been suggested to arise from disturbances in the balance between goal-directed and habitual control of behaviour, through dysfunctions in 'frontostriatal' circuits (Gillan and Robbins, 2014; Graybiel and Rauch, 2000). This dysfunction essentially makes patients 'stuck' in performance of their habits. This idea is supported by studies showing that OCD patients have reduced connectivity in frontostriatal circuits (Dong et al., 2020) and that the reduction in connectivity correlates with impairment on cognitive tasks that require goal-directed control of behaviour (Vaghi et al., 2017). Understanding these circuits could be key to finding new targets and developing new treatments for OCD.

The ability to learn and implement strategies for performance of a spatial sequencing task has been studied for both disorders in a sequence generation task. In this task four big squares were presented in a cross like arrangement (top, bottom, left and right) on a screen. Subjects were instructed to perform as many unique four-response sequences as possible. Each spatial location could only be selected once per trial, making the maximum number of sequences 24. After a first attempt, subjects were then trained to use a strategy. The strategy aimed to reduce the working memory load of the task, by dividing it into four bins of six responses, instead of one bin of 24 responses. For each bin, responding always started at one location, until all 6 responses had been performed. Thus, subjects were only required to remember their responses for one bin at a time. After strategy training, subjects were told to perform the task again, still with the goal of making as many unique sequences as possible.

OCD patients did not differ from healthy human participants or trichotillomania patients on the first performance or on strategy training. However, OCD patients, unlike the other groups, were unable to improve their performance from baseline after strategy training - indicating that OCD-patients have an impairment in implementing heuristic strategies (Chamberlain et al., 2006). Schizophrenia patients showed an impairment which extended to learning of, and thus subsequently, execution of the strategy, even though their original performance was identical to IQ-matched controls (Iddon et al., 1998).

On the CANTAB SWM task (presented in **Box 1.1**) there is consistent evidence that patients with schizophrenia show impairments (Badcock et al., 2005; Bartók et al., 2005; Elliott et al., 1998; Hutton et al., 2004; Joyce et al., 2005), it has even been reported that they are more impaired than frontal lesioned patients (Pantelis et al., 1997). The strongest effect across studies is an increase of between-search errors. A reduction in strategy score is also apparent in many studies, though not all. A meta-analysis of all available studies would be beneficial, but attempts have been inconclusive due to heterogeneity between studies (Levaux et al., 2007). An impairment in planning is also evident in the CANTAB SOC (see **Box 1.1**) (Badcock et al., 2005; Elliott et al., 1998; Hutton et al., 2004; Pantelis et al., 1997; Tyson et al., 2004a), this impairment has been demonstrated to extend beyond IQ reduction in patients (Lemvigh et al., 2020).

For OCD patients the results are more mixed. It has been reported that OCD patients are impaired at the CANTAB SWM task, by increased errors and impaired strategy use (Perna et al., 2019; Purcell et al., 1998), or by only performing more errors on the difficult stages (Chamberlain et al., 2007). Another study showed a very mild impairment - OCD patients had impaired strategy use in the first session, with no other impairments (Morein-Zamir et al., 2010). Studies have even found no impairments (Nielen and Den Boer, 2003). It has been suggested that disease heterogeneity could explain the discrepancies. Relevant to this, 'checkers' have been reported to have a stronger impairment, compared to other subtypes of OCD (Nedeljkovic et al., 2009). However, this effect was not replicated in a larger study (Dittrich et al., 2011). The difference between studies could also be related to different capabilities to compensate for the impairment (Henseler et al., 2008), or the differences could be related to symptom severity (van der Wee et al., 2007).

More consistent effects can be found on performance of the CANTAB SOC task. Early studies found impairments only related to thinking times (Purcell et al., 1998) and impairments related to increased latencies in generating new plans after an incorrect trial, even though accuracy remained intact (Veale et al., 1996). Using a version of the task, CANTAB One Touch Stockings of Cambridge (OTS), where subjects are required to internally estimate the number of moves required, instead of actually performing the moves, Chamberlain et al (2007) found that OCD patients required more attempts for correct responses. This finding was replicated and extended in a later study when it was demonstrated that the CANTAB OTS impairment in accuracy and slowed latency of OCD patients correlated with lowered resting state connectivity between right putamen and right dlPFC (Vaghi et al., 2017b). Further work has revealed that this is true also for activity during performance of CANTAB OTS and that the impairment extends to first-degree relatives, as opposed to healthy controls, indicative that this planning deficit is an endophenotype of OCD (Vaghi et al., 2017a).

Box 1.3 – Brief Introduction to schizophrenia

Schizophrenia is a debilitating psychiatric disorder with a range of emotional, behavioural and cognitive symptoms. Symptoms are divided into negative, positive and cognitive. Positive symptoms are changes in perception, delusions, hallucinations, altered speech etc. Negative symptoms are anhedonic symptoms, lack of motivation, lack of pleasure etc. Cognitive symptoms include dysexecutive functions such as attention and working memory. The prevalence of schizophrenia is thought to be between 0.4-0.7% over a lifetime (Saha et al., 2005). Schizophrenia is treated with antipsychotics, of which two generations exists, typical and atypical. Typical antipsychotics are antagonists of the D_2 receptor while atypical antipsychotics also act as antagonists for the $5HT_{2A}$ receptor and to a varying degree as an agonist for the $5HT_{1A}$ and antagonist for the D_2 receptor (Kusumi et al., 2015). Antipsychotic drugs generally treat positive symptoms well (Buchanan and Ball, 1998), even though some patients remain resistant (Conley and Kelly, 2001). Antipsychotics do however have only limited beneficial effects on cognitive symptoms (Mishara and Goldberg, 2004) and drugs that aim to increase the cognitive ability of schizophrenia patients would benefit patients (Gold, 2004), particularly as recovered cognition is important for functional recovery (Green et al., 2004, 2000).

Collectively, for schizophrenia, there is clear evidence for impaired performance of sequences that require intact ability to use strategy and generate behavioural plans. There is also strong evidence for similar impairments in OCD patients.

This is of particular interest because functional connectivity between caudate and the lPFC have been suggested as a biomarker for OCD (Dong et al., 2020). Similarly, resting state connectivity between caudate-vlPFC and putamen-dlPFC correlate, respectively, with impairments in cognitive flexibility and planning (Vaghi et al., 2017b). Dysconnectivity between lPFC and striatum can also be found in schizophrenia (Zhou et al., 2007) and altered connectivity between these areas correlate with impaired executive functioning (Quidé et al., 2013). Advancing our understanding of how these circuits operate to generate behavioural plans offers possibilities in developing novel psychological treatments. Understanding the neural modulation of these circuits offers possibilities for developing new pharmacological targets that decrease suffering of patients.

1.4. Chemical neuromodulation of PFC

So far in this introduction, frontal lobe functioning and performance of response sequences have been reviewed, both with an emphasis on the lPFC. However, the chemical neuromodulation of fronto-executive functioning has not yet been considered. This is particularly relevant for development of novel pharmacological treatment which might aid cognitive deficits in psychiatric disorders.

1.4.1. Neuromodulation of PFC by dopamine and serotonin

The most abundant neurotransmitters in the brain are glutamate (Fonnum, 1984) and GABA (Krnjević, 2004). GABA and glutamate are primarily fast acting signalling systems, under control by other neurotransmitters. This section will be limited in scope to the two neurotransmitters experimentally investigated later in this thesis, the indoleamine serotonin and the catecholamine dopamine. PFC (and subcortical areas) is innervated by ascending monoamine systems, originating from the brain steam or midbrain. These neurotransmitters acts as agonists on a set of receptors, five receptors for dopamine, from D_1 to D_5 (Missale et al., 1998), and fourteen receptors for serotonin, classified in families of $5HT_1$ to $5HT_7$ (Nichols and Nichols, 2008). The intracellular effect of these receptors varies, some are excitatory (e.g. $5HT_{2A}$ and D_1) while some are inhibitory (e.g. D_2 and $5HT_{1A}$). The expression of these receptors

also varies across the PFC. D_1 is more abundant and expressed across more cortical layers than D_2 (de Almeida et al., 2008; Lidow et al., 1991), while D_3 shows almost no expression in the PFC (Lévesque et al., 1992).

A role for prefrontal monoamines in the regulation of executive function was first shown for dopamine and working memory (Brozoski et al., 1979). They demonstrated that depleting dopamine in the dlPFC of rhesus macaques severely impaired performance of a spatial delayed alternation task. This impairment could partly be ameliorated by administration of dopamine agonist apomorphine or the biochemical precursor of dopamine, L-dopa. This sparked a lot of research into the role of dlPFC dopamine in working memory. Working memory performance in an oculomotor delayed-response task was shown to be sensitive to D_1 but not D_2 blockade (Sawaguchi and Goldman-Rakic, 1994). However, stimulating D_1 receptors also impair working memory (Zahrt et al., 1997), indicating that both too little and too much stimulation impairs performance. The dose-response relationship forms an 'inverted U-shape' (see, e.g., Cools and D'Esposito (2011) for review). Further research extended the role of prefrontal dopamine beyond working memory. Depletion of prefrontal dopamine in the marmoset monkey has been demonstrated to enhance performance of extra-dimensional shifts in a primate analogue of the WCST (Roberts et al., 1994). A follow up study revealed that the same depletion impaired performance of repeated intra-dimensional shifts, even though simple reversal learning was left intact (Crofts et al., 2001). The depletion caused an attentional dysfunction and it has been suggested that prefrontal dopamine acts to stabilise attentional representations (Robbins, 2005). The role for D_1 in working memory have been replicated in rodents, infusions of a D_1 antagonist impaired performance of a delayed response radial-arm maze task (Seamans et al., 1998). The function of D_1 has also been extended to include behavioural flexibility (Ragozzino et al., 1999) by showing that blockade of mPFC D_1 impairs shifting between two different strategies. A similar role for D_2 has also been established in behavioural flexibility, where D_2 blockade also impaired strategy shifting (Floresco et al., 2006). Later studies in primates have extended these findings by showing that separate intra-vlPFC infusions of D_1 (Puig and Miller, 2012) and D_2 (Puig and Miller, 2015) receptor antagonists impaired learning of new visual oculomotor associations and cognitive flexibility but left performance of already learnt associations intact. This empirical evidence generally fits well with a computational model of prefrontal dopamine,

the dual-state theory (Durstewitz and Seamans, 2008). The-dual state theory suggests that prefrontal D_1 and D_2 act in opposing fashion to guide behaviour. The D_1 state acts to stabilise representations for online maintenance through a high-energy barrier. The D_2 state is characterised by a low energy barrier and acts to flexibly shift between different representations. However, in the orbitofrontal cortex, the role of dopamine is less clear. Dopamine depletion of the OFC does not affect reversal learning (Clarke et al., 2007). However insensitivity to conditioned reinforcers and an inability to cease responding during extinction have been shown after OFC dopamine depletion (Walker et al., 2009b). Following serotonin depletion marmosets exhibited stimulus-bound responding on both visual discrimination and extinction but showed a strong bias towards initial visual stimulus preferences (Walker et al., 2009b). This suggests that OFC dopamine is important for associative processing of reward while OFC serotonin acts to inhibit responses to competing salient stimuli. This conclusion for serotonin needs to be considered collectively with findings that OFC serotonin depletion impairs reversal learning (Clarke et al., 2004) but not attentional set shifting (Clarke, 2005). This impairment is specific to inhibiting responses to a previously rewarded stimulus and not in approaching a previously unrewarded stimulus (Clarke et al., 2007). A role for serotonin in behavioural flexibility has also been confirmed in rodent studies, both $5HT_{2A}$ and $5HT_{2C}$ receptors play a role in mediating performance (Alsiö et al., 2015; Barlow et al., 2015; Boulougouris et al., 2008). However, systemic treatment with range of other serotonin agonists and antagonists have also been shown to affect cognitive flexibility (Nilsson et al., 2019).

The prefrontal dissociation on performance of reversal learning (dependent on an intact OFC) and set shifting (dependent on intact lPFC) is further highlighted by the neurochemical dissociation between the two. Previously in this Introduction, evidence was presented for the crucial role lPFC plays in behavioural planning. Very little is known about the neuromodulation of behavioural planning. Based on a dopaminergic role, and not a serotonergic role, in higher-order shifting, one could presume that lPFC behavioural planning is dependent on dopamine. However, extensive prefrontal dopamine depletion in the marmoset was without effect on performance of a spatial self-ordered sequencing task (Collins et al., 1998). Similarly, global prefrontal serotonin depletion was without effect (Walker et al., 2009a).

However, there is also evidence in favour of an involvement of both these neurotransmitters in planning. A high dose of the selective D_2 antagonist sulpiride impairs performance of both CANTAB SWM and SOC (Naef et al., 2017), similarly it also impairs a sequence generation task (introduced previously in the context of OCD and schizophrenia) (Mehta et al., 1999). This is further supported by findings that increasing dopamine levels by methylphenidate, a dopamine transporter inhibitor, improves performance of behavioural planning and sequence generation (Elliott et al., 1997). For serotonin the evidence is less clear, but exists nonetheless. Acute tryptophan depletion (ATD) is a dietary intervention used to study the cognitive and emotional effects of transiently lowered serotonin levels in humans. ATD is without effect on CANTAB SOC in healthy individuals (Murphy et al., 2002), but not in depressed patients (Elliott et al., 1996). However, these findings have been interpreted as an increased affective reaction to negative feedback of erroneous responses, rather than planning itself. More important is the finding that schizophrenia patients treated with atypical psychotics show different cognitive recovery. Patients treated with antipsychotics with a higher affinity to the $5HT_{2A}$ receptor demonstrate decremental planning performance over time, this is in contrast to patients treated with low $5HT_{2A}$ affinity antipsychotics (Tyson et al., 2004b). Also, LSD, a potent $5HT_{2A}$ and D_2 agonist has been reported to impair performance on the CANTAB SWM, this impairment being ameliorated by co-administration of a $5HT_{2A}$ antagonist (Pokorny et al., 2019).

The role of prefrontal dopamine and serotonin on behavioural planning needs further investigation. In particular, the role of specific dopamine and serotonin receptors will be key to understanding this complex behaviour. Priority targets among the dopamine receptors is the D_2 receptor and for serotonin the $5HT_{2A}$ receptor. If successful, it might open avenues to new pharmacological targets and guide clinical decisions related to already approved drugs.

1.4.2. Prefrontal D_2 and $5HT_{2A}$ receptors

Studies on D_2 and $5HT_{2A}$ distribution in the vlPFC are limited, but evidence is available from rodent mPFC and primate dlPFC, **Fig. 1.3** illustrates the expression of $5HT_{2A}$, D_2 and $5HT_{1A}$ receptors in PFC Layer V micro circuitry. There is consensus between findings in the rat (Santana et al., 2009) and primate (Lidow et al., 1998) that D_2 receptors are primarily expressed in layer V pyramidal neurons. During performance of a delay-related working

memory task, D_2 receptors in layer V of dlPFC are not required for maintaining information during delay, but activity of these neurons are related to responses in the task (Wang et al., 2004). A similar response related firing has also been found in the vlPFC (area just ventral of the principal sulcus) during associative visuomotor learning (Puig and Miller, 2015). The response related firing has been suggested to be processing of motor feedback (Wang et al., 2004). If feedback is received that current behavioural output is incorrect, inhibitory D_2 receptors would be able to suppress pyramidal output and allow for behaviour to be corrected. Dysregulation in these processes could lead to impairments in correcting erroneous responses.

$5HT_{2A}$ receptors are more widely distributed across the layers of PFC, but are primarily expressed in layers III and V pyramidal neurons (de Almeida et al., 2008) and layer V and VI fast-spiking interneurons (Puig and Gulledge, 2011). The $5HT_{2A}$ receptors located on pyramidal neurons are found on the apical dendrite, close to the soma (Jakab and Goldman-Rakic, 1998). Mechanistically these receptors have been suggested to amplify excitatory

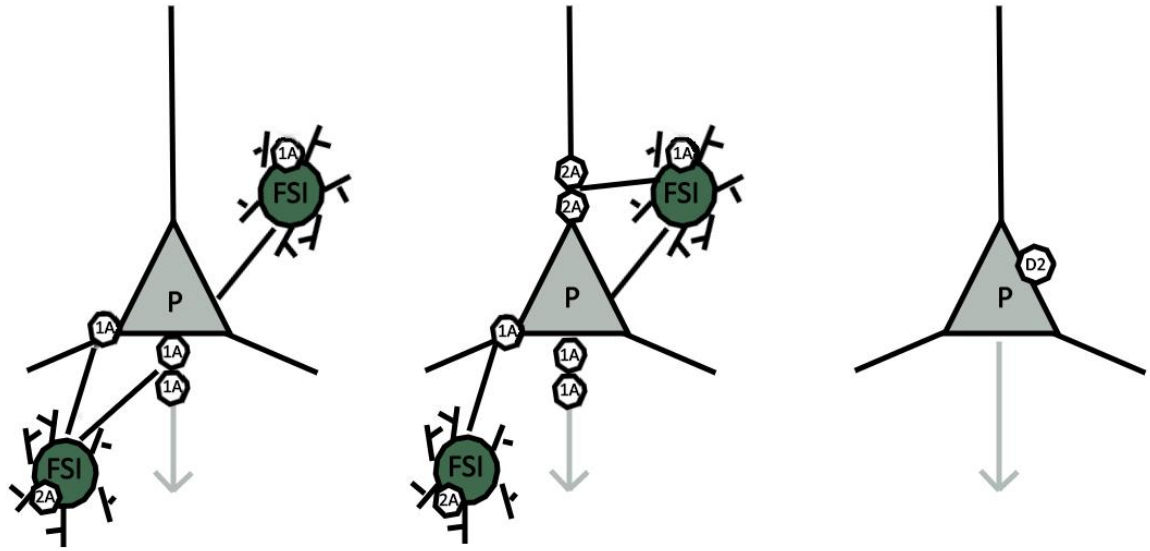


Figure 1.3 Locations of D_2, $5HT_{2A}$ and $5HT_{1A}$ receptors within PFC layer V microcircuitry. $5HT_{2A}$ receptors (labelled 2A) located on pyramidal neurons (labelled P, coloured grey) are found on the apical dendrite and act to excite pyramidal cells. $5HT_{1A}$ receptors (labelled 1A) are located on the axon initial segment and on dendrites of pyramidal cells and act inhibitory on pyramidal cell output. $5HT_{2A}$ and $5HT_{1A}$ receptors also have opposing effects on fast-spiking interneurons (labelled FSI, coloured dark green), where $5HT_{2A}$ act to excite interneurons, while $5HT_{1A}$ have inhibitory actions. Dopamine receptor 2 (labelled D2) are primarily found on pyramidal cells and suppress pyramidal cell firing. Image adapted from Puig and Gulledge (2011).

synaptic currents (Marek and Aghajanian, 1999). The deeper layer $5HT_{2A}$ interneurons target the soma of pyramidal cells (Puig and Gulledge, 2011). Evidence of a behavioural effect following lPFC administration of $5HT_{2A}$ agonists or antagonists are scarce, but a complex role for $5HT_{2A}$ in working memory has been demonstrated. $5HT_{2A}$ are important for spatial tuning of cells in dlPFC and have been interpreted as a mechanism to facilitate representations of sensory inputs and allocate attention (Williams et al., 2002). To fully understand neural modulation by an antagonist, consideration must also be taken to the finding that a majority of $5HT_{2A}$ expressing pyramidal cells in the PFC layer V also express $5HT_{1A}$ receptors (Amargos-Bosch, 2004). These receptors are located on the axon initial segment, where they act to inhibit pyramidal firing (Cruz et al., 2004). However, these receptors are not co-expressed on fast-spiking interneurons (Puig and Gulledge, 2011). The interplay between $5HT_{2A}$ and $5HT_{1A}$ pyramidal and interneurons is complex but they both act to regulate pyramidal output. The overall effect of treatment with a $5HT_{2A}$ antagonist, has been demonstrated to be decreased pyramidal output (Araneda and Andrade, 1991; Beique et al., 2007; Puig et al., 2003).

1.5. Experimental rationale and plan of the thesis

In this Introduction, evidence has been presented for a specific role of lPFC in organising higher-order behaviour and vlPFC was highlighted as a region of key importance to understanding cognitive deficits in disease. A role for vlPFC in spatial self-ordered response sequencing has been established in both humans and primates (Owen et al., 1996; Walker et al., 2009a), however more information is needed on the nature of the involvement, especially because of the previous emphasis on the dlPFC rather than the vlPFC. Findings from human, however, are primarily correlative, mainly from neuroimaging, and causal evidence is based on permanent lesion studies, where recovery and compensatory mechanisms, as well as the diffuse and non-specific nature of the lesions, might be of concern. Further causal investigation is necessary, particularly on the behavioural specificity and chemical neuromodulation, of which very little is known. Further investigation into the neural circuitry in relation to the vlPFC, is also required.

The experiments making up this thesis set out to investigate the contribution of vlPFC in performance of self-ordered spatial response sequencing. The causal role of vlPFC will be investigated in a modified version of the spatial self-ordered sequencing task (Collins et al.,

1998; Walker et al., 2009a). Novel task modifications were also developed to investigate the behavioural specificity of any impairment. In particular, much previous work using this type of behavioural procedure can be interpreted in terms of impairments in spatial working memory rather than control of response sequencing per se. One of the aims of this thesis was to try to minimise working memory requirements. In addition, I sought to contrast performance on variable sequences versus a fixed sequence because of the hypothesis that the vlPFC is important for behaviour which require performance to be flexible and adaptive, as opposed to organising behaviour performed as a learned skill.

These findings are followed up by investigations into the neuromodulation of vlPFC, of which very little is known. Previous findings in the marmoset have demonstrated that both serotonin and dopamine depletion within the PFC are without effect of self-ordered response sequencing. However, drugs targeting these systems have been demonstrated to affect planning. The depletion studies were not total and functional recovery or compensatory mechanisms are possible. Thus, I also sought to investigate if specific dopamine and serotonin receptors within the vlPFC, play a role in performance of self-ordered sequences.

In this introduction, a broad neural network was presented for performance of sequences. However, it is not clear which vlPFC projections are important for a proposed role of vlPFC in planning. Thus, I sought to explore the role of one possible target of vlPFC projections, the caudate nucleus, a target selected based on a demonstrated role in action sequencing and also due to the involvement of frontostriatal circuits in response sequencing deficits in disease.

In Chapter 3, the causal role of vlPFC in spatial self-ordered response sequencing was investigated by selective transient inactivation of this region using microinfusions. Intracerebral microinfusions through surgically implanted cannulas are a validated tool, previously used in rodents (see e.g., Boulougouris and Robbins, 2010; Floresco et al., 2006) and marmosets (see e.g., Clarke et al., 2015; Jackson et al., 2019). However, this chapter will present the first investigations made on local cerebral infusions into the vlPFC on performance of spatial response sequences. This will greatly extend previous findings that show that chronic excitotoxic lesions of vlPFC impair performance of spatial response sequences (Walker et al., 2009a). Previous investigations used a delay component, which were removed in the current study. This drastically decreased the working memory

component of the task and allowed investigations on action sequencing with minimal working memory, thus, excluding some alternative interpretations of findings. Further to this, a novel modification of the task was developed to allow investigations into the behavioural specificity of inactivation. This modification allows for comparisons of the causal role of vlPFC in both variable and fixed self-ordered response sequences. Also, concerns over functional recovery or compensatory mechanisms can be overcome using transient inactivation, rather than excitotoxic lesions.

In Chapter 4, investigations were made into the chemical neuromodulation of vlPFC on performance of variable self-ordered response sequences. The effect of local infusions of a $5HT_{2A}$ and, separately, a D_2 antagonist were investigated. Very few studies have selectively infused dopaminergic and serotonergic agents selectively into the vlPFC and to my knowledge no such study has ever been performed for a spatial sequencing task. The findings will build on the limited evidence from non-human primates of the role individual receptors play in the PFC and extend current understanding of the chemical neuromodulation of the prefrontal cortex.

Lastly, in Chapter 5 investigations were made into the neural circuitry responsible for performance of both variable and fixed self-ordered response sequences. An area of the caudate with input from the vlPFC was investigated. An AMPA-receptor antagonist was infused selectively into the caudate, with the goal of blocking glutamatergic input to the region. This is an important first step in mapping the neural circuitry involved in performance of variable and fixed response sequences. Investigations of this circuitry have considerable relevance for impairments in executive functioning in psychiatric disorders (Quidé et al., 2013; Vaghi et al., 2017b).

Together these experiments were designed to investigate the function, behavioural specificity, neuromodulation and neural circuitry of vlPFC in performance of spatial self-ordered response sequences.

2. General Methods

2.1. Subjects and housing

Eight common marmosets (*Callithrix Jacchus*), four female and four males, were the subjects of the experiments described in this thesis. For experimental allocation, please see **Table 2.1**. All marmosets were bred on-site in the University of Cambridge Marmoset Breeding Colony. They were removed from their family units around 18 months of age, at the time at which they naturally begin to exert greater dominance and begin to unsettle the family group. The marmoset holding rooms were constantly kept at 24 °C with a relative humidity of 55%. Holding rooms were gradually illuminated from 7.30 to 8.00 and gradually dimmed from 19.30 to 20.00, giving a 12 h light/dark-cycle with 30 minutes of dusk/dawn. Animals were kept in custom built cages (Techniplast UK Ltd., Kettering, UK) in male/female pairs, all males were vasectomised before starting an experiment. Each cage contained a food tray, a nest box, wooden platforms at different heights and a variety of enrichments. Animals were both food and water restricted during testing days (Monday - Friday) to increase engagement in behavioural testing. Animals were given a nutritionally complete diet. On Fridays and Saturdays animals were given fruits, rusk, sandwich with boiled egg and supplemented with addition of multivitamins and D-vitamin. On Sundays to Thursdays animals were kept on a calorifically equivalent but less varied diet, consisting of pellets and carrots or orange. Animals had restricted access to water during testing days, water was freely available for 2 h in the afternoon from 15.30 to 17.30. Animals had *ad libitum* access to water from Friday afternoon to Sunday evening. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 as amended in 2012, under project licences 70/7618 and P09631465. In addition, the University of Cambridge Animal Welfare and Ethical Review Body (AWERB) provided ethical approval of the project licence and its amendments, as well as individual studies and procedures via delegation of authorization to the NACWO for individual study plans.

			Cannulation sites		Chapter 3			Chapter 4		Chapter 5	
Subject number	Sex	Subject	vlPFC Cannula	Caudate Cannula	Variable 4-block spatial self-ordered sequencing task	Variable 1-block spatial self-ordered sequencing task	Fixed 1-block spatial self-ordered sequencing task	$5HT_{2A}$-R Antagonism	D_2-R antagonism	Variable 1-block spatial self-ordered sequencing task	Fixed 1-block spatial self-ordered sequencing task
1	M	Runner	✓		✓			✓	✓		
2	M	Kami	✓		✓			✓	✓		
3	F	Baku	✓		✓			✓	✓		
4	M	Yoda	✓		✓						
5	F	Vader	✓		✓			✓	✓		
6	F	Figrin	✓	✓		✓	✓			✓	✓
7	M	Skywalker	✓	✓		✓	✓			✓	✓
8	F	Anakin	✓	✓		✓	✓			✓	✓

Table 2.1 Experimental allocation of subjects. Table showing the subject number, gender, subject name, cannulation sites and the task and chapter allocation. -R denotes receptor.

2.2. Behavioural testing apparatus and transport box

All behavioural testing was performed in a custom-built testing apparatus located in a separate room from the marmoset holding rooms. To allow transportation to the testing apparatus, animals were trained to enter a custom-made Perspex transport box (Biotronix, Cambridge, UK). To allow animals easy access to the transport box, one of the longer sides consisted of a metal door that moved in and out from the side of the box. The opposite side had six small air holes and a larger hole that was used to give the marmoset treats as an incentive to enter the box. Once a marmoset had entered the box, the researcher carried the box over to the testing room using the attached handle. The marmoset remained in the box during testing. The box slotted into the testing apparatus and locked into place using a latch located on of the short side of the box.

To transfer the marmoset to the testing apparatus, the door of the transport box was removed, allowing access to a touchscreen and a reward licking-spout (Campden Instruments, Loughborough, UK). To prevent accidental responses to the screen by the tail, subjects had to reach between vertical bars to access the screen and the licking spout. Liquid reward was given through a licking spout and licks were detected by an IR-sensor on the spout. The liquid reward, banana milkshake (Nestlé), was the same for all animals of the study and throughout all stages of testing. It was made fresh every morning and kept on ice during testing. A set of speakers inside of the box, out of sight of the animals, allowed playback of auditory stimuli during testing. The chamber had LED-stripes attached to the top-inside of the box to create a house light. Three video cameras were mounted inside of the cage to allow monitoring of the animal from outside of the testing chamber on a personal computer. The touchscreen, speaker, house light and reward spout were all connected to the same personal computer and could be controlled by the application MonkeyCantab (R.N. Cardinal) using the Whisker control system (Cardinal and Aitken, 2010).

2.3. Pre-operative behavioural training

The first goal with early pre-operative testing was to train the animals on how to use the touchscreen and take liquid reward. **Table 2.2** shows an overview of early training. All subsequent testing aimed at training the subjects on a spatial self-ordered sequencing task where the animal had to respond to two or three identical stimuli presented on the screen.

	Habituation			Reward training		Touch training	
Stage component	Habituation to transport box	Familiarisation of liquid reward	Habituation to test box	Stage 1	Stage 2	Big square	Small circle
Brief description	Gradual habituation to transport box in home cage	Familiarisation with liquid reward (banana milk) through a syringe in the home cage	Animals were transported to test box and habituated to being inside of the box	Liquid reward was freely available in test box through a licking spout	Liquid reward available on an 8s on/off schedule. Reward accompanied by CS+	Response to a big square presented in the centre of the screen yielded reward	Response to a small circle, presented randomly each trial between 8 locations gave reward

Table 2.2 Early pre-operative behavioural training. Table presents an overview of the three stages of early training; Habituation, reward training and touch training. Each training steps contains smaller components which have a brief description. After passing the early stages of training, animals were moved onto the training protocol for the spatial self-ordered sequencing task, see **Table 2.3.**

The task required animals to learn an abstract rule “press each stimulus once and once only”. Animals were free to respond to the stimuli in a self-ordered fashion and stimuli changed spatial position on every trial to maintain the requirement for flexible responding.

Animals were first habituated to the transport box. The researcher sat next to the home cage, opened the door and encouraged marmosets to enter the cage for rewarding 'treats'. When the marmosets were comfortable inside the box, the door was closed for short sessions followed by the transport box gradually being moved further away from the cage with the animal inside. Once the marmoset would readily take treats while in the box, it was transported to the test room and given treats inside the behavioural testing apparatus. During this stage animals were also familiarised with liquid reward in the home cage. After the animal readily accepted liquid reward from a syringe in the home cage, they were trained to lick at the spout inside of the testing apparatus in two stages. During stage one, animals were placed in the box in front of the touchscreen with the reward continually being delivered. To encourage animals to approach it, the spout (together with the adjacent vertical bars) was covered in small sticky pieces of marshmallow. When an animal tried to remove the treats, they would notice and consume the liquid reward. Stage 1 was completed once animals readily consumed the freely available reward. In stage 2 reward was freely available until 10 licks had been detected, followed by a schedule of 8-seconds on and 8-seconds off. The 8-seconds on was accompanied by an auditory conditioned stimulus (CS+), ‘birdsong’. This auditory stimulus was presented for the duration of the reward and accompanied it in all

subsequent stages of pre- and post-operative testing. After successful reward familiarisation, animals began touchscreen testing. Animals were presented with a large yellow square as the visual stimulus in the centre of the touchscreen and in front of the licking spout. After a successful response (touch) directed to the stimulus, the square disappeared, the CS+ began and reward was delivered. After reward delivery there was a 3 second inter-trial interval (ITI) until the stimulus re-appeared. At this stage, animals were unfamiliar with responding to a screen and so to encourage its exploration, marshmallows were attached to it. For the first few sessions, marshmallows were placed on the screen, in front of the stimulus. A few smaller pieces of marshmallows were accessible to the marmoset and one whole marshmallow was placed behind translucent tape. The accessible marshmallow pieces were removed between sessions, once animals showed interest in the screen. The taped marshmallow was removed once animal started taking liquid reward while ignoring the marshmallow. In the next stage the marshmallow was removed, and animals had to respond to the stimulus and take reward at least 20 times in a 20-minute session to pass criterion. The next step of training was very similar to the previous stage, with the same criterion of 20 trials in 20 minutes, but the visual stimulus was replaced with a smaller yellow circle. Once the animal selected the stimulus, visual feedback was provided by the stimulus disappearing and a brief presentation of a smaller circle in the same position for 0.1 seconds. The CS+ then began playing and a big yellow square was presented over the entire screen for 0.1 seconds, to further signal the end of trial and reward availability. New stimulus positions were also introduced. Stimulus positions were randomised every trial based on an 8-position grid**.** The small yellow circle stimulus, stimuli positioning, and visual response feedback would remain constant throughout all subsequent stages of pre- and post-operative testing. Once animals had readily responded to the stimulus in all the different positions, a second identical stimulus was presented simultaneously with the first, and animals only received reward once they had responded to both stimuli once and once only (see **Fig. 2.1** for illustration of temporal events on a three circle trial). No order was imposed, animals were free to respond to stimuli in a self-ordered fashion. After the selection of one stimulus, it disappeared only to return after the 'vanishing time'. Only the selected stimulus disappeared, and animals could continue responding during this time. If a subject re-selected a stimulus after it had re-appeared the trial was aborted and re-started after an ITI. Animals began training with a long vanishing time, to increase the time between stimulus selection and opportunity for error. Training was

done in gradual steps, see **Table 2.3** for details, where the number of concurrent stimuli was slowly increased and vanishing time decreased, until animals could perform two-stimuli and three-stimuli trials with a 0.5 second vanishing time.

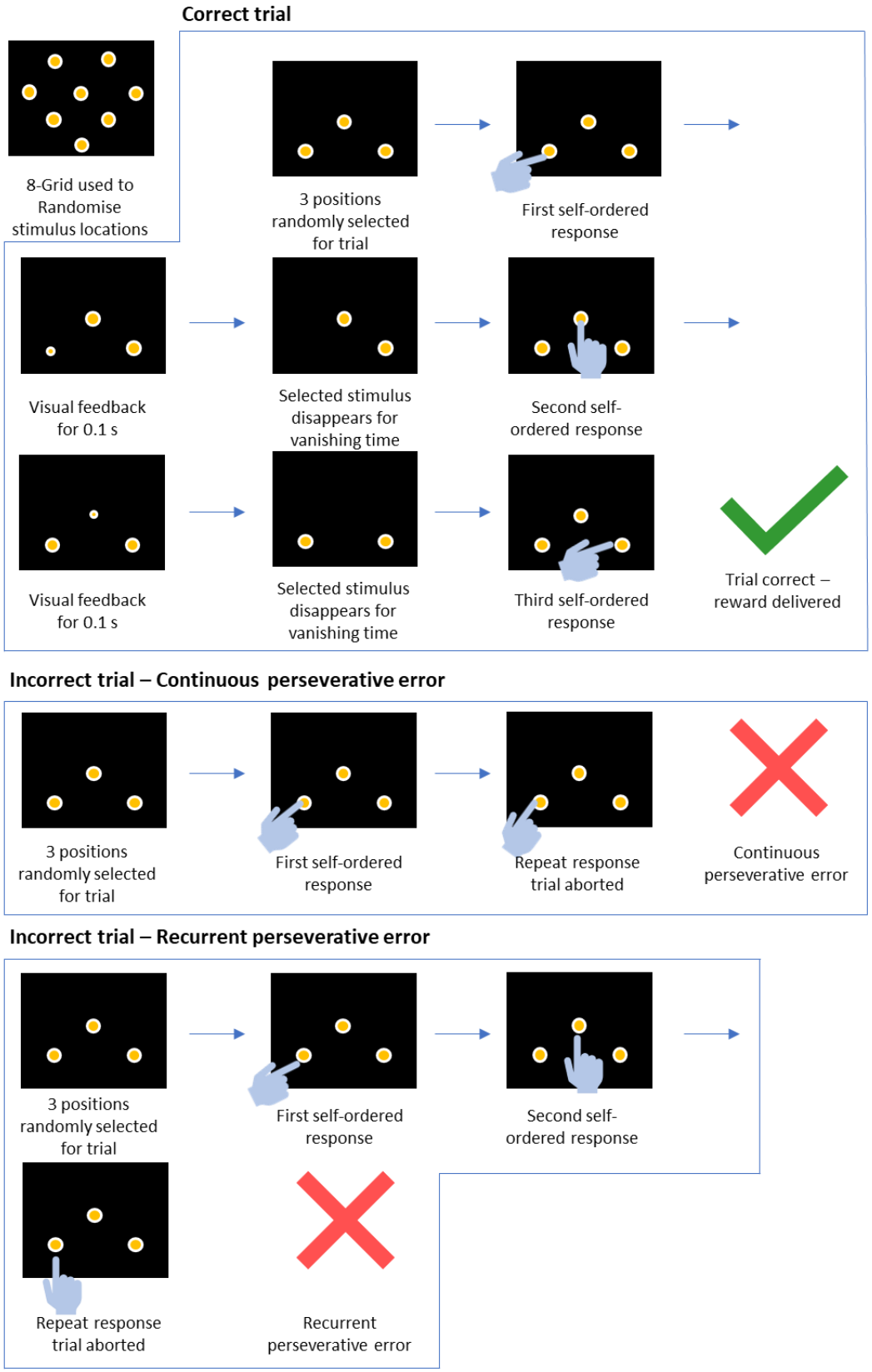


Figure 2.1 Illustration of temporal events on a given trial in the spatial self-ordered sequencing task. Figure illustrates correct and incorrect responding for a 3-circle trial. For every trial the stimuli locations are selected randomly based on an 8-grid and subjects are free to respond to presented stimuli in any order. Once a response has been made a short visual feedback is presented and stimulus disappears during vanishing time (both not shown for incorrect trials). Animals can continue responding during the vanishing time. If no response is repeated, trial is counted as correct and reward delivered. If on a given trial the previous response is repeated, it is counted as a continuous perseverative error, while if a response is made in-between repetition it is counted as a recurrent perseverative error.

Training Stage	1	2	3	4	5		6		7		8	
Name	1-circle	2-circle (vt=5)	2-circle (vt=3)	2-circle (vt=1)	2-circle (vt=1)	3-circle (vt=3)	2-circle (vt=1)	3-circle (vt=1)	2-circle (vt=0.5)	3-circle (vt=1)	2-circle (vt=0.5)	3-circle (vt=0.5)
Number of Stimuli	1	2	2	2	2	3	2	3	2	3	2	3
Maximum session length (min)	20	20	20	20	20	20	20	20	20	20	20	20
Maximum trials	-	20	20	20	20	20	20	20	20	20	20	20
Inter-trial interval (s)	3	3	3	3	3	3	3	3	3	3	3	3
Reward Duration (s)	8	8	8	8	8→6→5	8→6→5	5	5	5	5	5	5
Waiting time (s)	900	900	900	900	60	60	60	60	60	60	60	60
Vanishing time (s)	N/A	5	3	1	1	3	1	1	0.5	1	0.5	0.5
Criterion to pass	20 responses	≥ 70% correct	≥ 70% correct	≥ 70% correct	≥ 70% correct	≥ 70% correct	≥ 70% correct	≥ 50% correct	≥ 70% correct	≥ 50% correct	≥ 70% correct	≥ 50% correct

Table 2.3. Training protocol for the spatial self-ordered sequencing task. After animals had passed initial touchscreen training, they had to pass each individual training stage before they could undergo cannulation surgery. Number of concurrent presented identical stimuli were gradually increased while the vanishing time was gradually decreased. The vanishing time is the time a stimulus disappears after selection, before re-appearing. Animals could continue responding while the previously selected stimulus was hidden. Once animal had passed a training stage, they were moved on to the next step the following test session. In training stage 5 reward length was gradually decreased and animals had to pass training on all reward lengths until they could move on to training stage 6.

2.4. Surgical procedures

2.4.1. Pre-surgical procedures

On the day before surgery animals on water-restriction were allowed full access to water and weighed to ensure weight was stable. Surgery was also set up on the day before to ensure sufficient autoclaved equipment and consumables were available. Surfaces in surgery and pre-surgery, including the stereotaxic frame and arms were wiped down with alcohol and anistel (Tristel Solutions Ltd, Cambridgeshire, UK).

Animals were weighed again on the morning of surgery. If weight had dropped more than 5%, surgery was postponed. Water restricted animals had full access to water before surgery but did not have food 12 hours prior to surgery to reduce adverse effects while recovering from the anaesthesia. Animals were pre-medicated with 0.1 ml of 100mg/ml of ketamine (Ketavet, Henry Schein, USA) i.m and placed in an incubator for around five minutes until the animal was sedated. Once out of the incubator animals were immediately put on a heat mat to allow normal body temperature to be maintained. The top of the head was shaved to allow incisions to be made. Hands and feet were also shaved to allow for readings from a pulse oximeter. Animals were also administered a long lasting non-steroidal anti-inflammatory drug, Carprofen (Pfizer, Kent, UK), 0.03ml of 50 mg/ml given s.c.

2.4.2. Anaesthetic procedures

Animals were moved from the pre-surgical area to the surgical suite and kept on a heat mat throughout surgery. Anaesthesia was maintained using a mixture of vaporised isoflurane (Novartis animal health, UK) and O_2 , controlled by an anaesthetic machine (Compact Anaesthesia Systems, VetTech Solutions Ltd., UK). Anaesthesia was induced using a facemask (4% Isoflurane in 0.7 L/min O_2). When an animal reached unconsciousness, as measured by lack of muscle tension, it was intubated. For intubation, one experimenter held the animal by its cheekbone. A second researcher applied lidocaine (IntuBeaze, Dechra, Shrewsbury, UK) to the epiglottis and inserted an endotracheal tube. The endotracheal tube was then connected to the anaesthetic machine and isoflurane levels lowered to 2.25% in 0.3 L/min O_2. The animal's body temperature was controlled throughout surgery by a rectal thermometer probe, protected by a disposable lubricated plastic sheet. Vital signs were monitored

throughout the surgery by a combined pulse-oximetry and capnograph (MicroCap Handheld Capnograph, Oridion Capnography, USA). Heartrate, oxygen saturation, respiratory rate and body temperature were constantly monitored and noted on a sheet every five minutes so that changes in vital signs over time could be observed. Anaesthetic levels were constantly evaluated, and adjustments to the level of anaesthesia were made based on changes in vital signs. To prevent dehydration, animals were given 0.1ml of warm saline every 60 minutes. Animals were rotated between lying on their front, left and right every 20 minutes in order to stimulate blood flow.

2.4.3. Cannulation surgery

Two different cannulation surgeries were performed for the experiments described in this thesis. For the experiments described in chapter 4, and in animals running the 4-block task in chapter 3, one set of two double cannula were implanted targeting vlPFC (Brodmann area 47/12). Animals running the 1-block task in chapter 3 and chapter 5 had two sets of two cannulas; one set of double cannulas targeting vlPFC and the other set of single cannulas targeting the caudate nucleus. The present author was responsible for setting up surgery, holding for intubation, monitoring and assisting during surgery as well as post-surgical monitoring and drug administration. Professor Angela Roberts performed the early parts of surgery, up until depth checks and locationing of cannulation targets. After the depth checks another researcher performed the rest of the surgery. Dr. Nicole Horst performed surgery, post depth-check, on Subject 1-5 and Dr. Hannah Clarke performed subjects 6-8.

Animals were placed into a stereotaxic frame (David Kopf Instruments, California, US) following intubation. Ear-bars were fitted into the ear canal of the marmoset and adjusted until head was centred in the frame. The ear bars were screwed tightly into the frame to prevent lateral movement during the surgery. Eye bars were fitted into the supraorbital foramen of the eye sockets and a mouth bar place against the roof of the mouth. Lacri-lube (Allergan, Bucks, UK) was applied to the eye lids to prevent drying of the eyes. Great care was taken not to compress the animal's tongue when fitting the mouth-bar. The animal’s body was covered with an op cover (Jørgen Kruuse A/S, Denmark). The top of the head and the surrounding area was disinfected with a ChloraPrep (Invicta Animal Health, UK) chlorohexidine stick and an Ioban (3M Healthcare) sheet applied. A midline incision was made

from front to back of the top of the scalp to expose the skull. An eye speculum was put in place to retract the skin around the skull. To allow for correct cannula placement the canula coordinates were adjusted based on a brain depth check (Dias et al., 1997). A reading was taken on the ear-bars (inter-aural line) and used for the anterior-posterior (AP) coordinate 0 (positive values in the anterior direction). A probe was moved to +17.5 and the skull was drilled to expose the sagittal sinus. A reading was taken on the sinus to provide the latero-medial (LM) coordinate 0 (positive values on the animal's left side). A depth check was made at +17.5 AP, +1.5 LM by taking a measurement of the brain surface with the probe and then carefully lowering the probe through the brain until it reached the base of the skull where a second measurement was taken. The difference between these readings needed to be between 5.8-6.8 mm. If the reading was too high another reading was taken further forward (+0.5-1.0) until the depth fell within the 5.8-6.8 range. If the reading was too low, it was re-adjusted in the same manner, but moved back. These same adjustments (if needed) were then applied to the target AP coordinates for all cannulas.

For the vlPFC cannulation a further depth check was needed to determine LM coordinates. The cannulas used to target the vlPFC were a double cannula, 26-gauge guides (Plastics One, Virginia, USA), cut 6 mm below the pedestal, with 1mm between the centre of the guides in the AP direction. The guide was inserted at an angle between 8-10 degrees (for details on individual subjects see experimental chapters) with the top of the cannula angled toward the midline. The depth-check was made at a matching angle of 8-10 degrees at AP +17.25 (midway between guides) and LM +-5.8. The reading should have been within the range of 3.0-4.5; if not, the LM coordinate was adjusted by moving medially or laterally as required. Once the LM coordinate provided the required depth, holes for the cannula guides were drilled at AP ±0.5 from the depth check hole, and the process repeated for the other hemisphere. Placements for 4 skull screws (PlasticsOne) were then determined and holes drilled using a manual drill. A layer of polymer adhesive (Superbond C&B; Sun medical CO. Ltd., Shiga, Japan), was applied to the skull. This layer helps protect the skull from the dental cement used later. The screws act to stabilise the head mount by adhering the dental cement to the skull. The cannula was attached to the stereotaxic arm and moved to determined coordinates, where holes had already been drilled. Once at the coordinates the cannula was angled to the desired angle (8-10 degrees). A surface reading was taken for the posterior

guide and the cannula was lowered until it reached 1.2mm above the base of the skull, calculated from the vlPFC depth check. The cannula was then mounted onto the skull and screws using dental cement. After the dental cement had dried a dummy injector (PlasticsOne) was inserted to maintain patency of the guide and a metal dust cap screwed on. Once cannulas had been cemented in place bilaterally, animals with only vlPFC cannula were now ready to have incision sutured while animals with vlPFC and caudate cannula had to undergo caudate cannulation before surgery could be finished.

Animals allocated for chapter 5 and a subset of the animals used in chapter 3, had two further cannulae implanted, targeting the caudate nucleus. The caudate cannulae were single guides (PlasticsOne), 26 gauge, cut 7 mm below pedestal. The AP coordinates for the caudate were +11 AP (adjusted from depth check if required) and +- 3.3mm LM. The target cannula depth, based on a marmoset brain atlas (Paxinos et al., 2012) was determined to be 12.7 mm up from the ventral base plate, while the top of the skull was 17 mm from ventral baseplate. This proportion (12.7/17) was used to adjust the cannula depth. Holes were drilled at +11 mm AP and +-3.3mm LM. The cannula was mounted to the stereotaxic arm and the arm moved to a separate plate which allowed a ventral baseplate reading. After the baseplate reading was taken, the arm was moved back to the stereotaxic frame, a surface reading was taken, and a new target could be calculated using the average value from the left and right side. For example, if the average distance from surface to ventral baseplate was 19.1 in an animal. 19.1 would be multiplied by (12.7/17) to obtain the new target of 14.26 mm from the ventral baseplate. After the new target depth had been calculated, the cannula was lowered into the brain and attached with dental cement onto the screws already mounted during earlier steps of the cannulation. Once the cement had dried, a plastic cap with dummy injector (PlasticsOne) was screwed onto the guides.

At this stage the Ioban cover was removed and the incision cleaned and sutured shut around the implant using 3.0 vicryl (Ethicon Inc, Puerto Rico). The animal was administered 0.18 ml of 3.8 mg/ml dexamethasone (0.09 ml injected into each quadricep) (Aspen Pharma Trading Ltd., Ireland) to prevent post-surgical inflammation. Isoflurane was turned off, but oxygen was provided until the animal could maintain oxygen saturation independently. They were then placed into an incubator and monitored closely for the next few hours. Animals were returned to their home-cage once they had recovered from anaesthesia, normally after 2-3

hours in the incubator. The marmoset was administered analgesia (0.1 ml of a 1.5 mg/ml solution of meloxicam; 'Metacam', Boehringer Ingelheim, Germany) orally every morning at 8.00 for the next three days and monitored several times daily for the next few days. Animals received water ad libitum and extra food for at least a week following surgery to enable full recovery.

2.5. Drug treatment

2.5.1. Drug infusion procedure

Intra-cerebral drug infusions were carried out in a designated 'minor procedures' room, separate from the home cage and behavioural testing apparatus. The surfaces in the minor procedures room were wiped down with anistel and alcohol before infusions. The entire infusion set-up, apart from the infusion pump, was sterile, see **Fig. 2.2**. Sterile swabs were placed on the pump so the experimenter could control the pump without violating sterility. Injectors were connected to a 0.3 mm diameter tubing (VWR International Ltd., UK) fitted to a 10 µl gas tight Hamilton syringe (Hamilton, Reno, NV, USA) via pfta tubing. The entire system

Figure 2.2 Setup for central infusions. The image illustrates the infusion set-up in the minor procedures room. To the left in the picture is the infusion pump. Circle 1 illustrates a sterile swab placed on the setup to allow for control of the pump without violating sterility. The big green square is a sterile field with the required equipment. Circle 2 illustrates a syringe filled with saline, connected to an injector. The tubing runs along the sterile field with an injector placed in drug/vehicle solution (Circle 3).

was filled with saline before a small air bubble was drawn into the tubing. The injector was submerged into a drug solution, which was drawn into the system. The air bubble was marked with permanent marker and movement of the air bubble was confirmed post infusion to confirm that drug solution had moved appropriately and hence had been administered.

For the infusion procedure, the awake marmoset was gently held by another researcher. The experimenter removed the dust cap and dummy injectors from the guide and wiped the top of the guide with injection wipes. The injector was inserted into the cannula and the pump turned on for one or two minutes, depending on the target volume to be administered. The rate of infusion used for all the experiments in this thesis was 0.3 or 0.5 ul/minute, depending on drug and experiment, see methods in experimental chapters. After infusion the injector was left in place to allow diffusion of solution for one minute. After injector removal, sterile dummy injectors were inserted into the guide and a sterile dust cap screwed on. Animal was returned to their home-cage before being tested. The time spent in the home-cage after infusion before testing, 'wait-time', differed between drugs used in this experiment, see **Table 2.4**. Rationale for wait-time for each individual compound is given in the relevant experimental chapter.

Drug	Supplier	Mechanism	Wait-time
CNQX	Tocris, UK	AMPA R antagonist	10 minutes
M100907	Sigma-Aldrich, MI, USA	5-HT_{2A} R antagonist	12 minutes
Muscimol / Baclofen	Sigma-Aldrich, MI, USA	$GABA_A$ / $GABA_B$ R agonist	25 minutes
S-(-)-Sulpiride	Sigma-Aldrich, MI, USA	Dopamine R D2 antagonist	10 minutes

Table 2.4 Drugs and their corresponding wait-time. List of drugs centrally infused in the experiments described, their supplier, molecular mechanism of action and wait-time. Wait time was the time the animal spent in the home-cage following infusion before being tested on a behavioural task.

2.6. Cannula maintenance

Cannula maintenance was carried out once weekly to maintain patency. Cannula caps and dummy injectors were replaced with autoclaved caps and dummies. The awake marmoset was gently restrained and taken to the 'minor procedures room' by another researcher. Caps and dummy injectors were removed and guides wiped down with injection wipes, before caps and dummies were replaced with autoclaved replacements. The area surrounding the implant was also inspected and cleaned using 70% ethanol on applied to sterile cotton buds. The guide locations used in this experiment were more anterior than previous experiments and animals required extra care as they easily developed infections around the implant.

2.7. Euthanasia and histological analysis

Histological analysis was needed to confirm the placement of cannula. To decrease suffering, animals were pre-medicated with ketamine and placed into an incubator for five minutes. Once animals were in a semi-conscious state they were taken out of the incubator and put onto a fleece. Animals were then injected with 1ml of 200mg/ml solution of pentobarbital IV (Dolethal; Merial Animal Health, Essex, UK). Loss of heartrate was confirmed using a stethoscope before animal was perfused transcardially with 300ml 0.1 M phosphate buffered saline, following 300 ml 10 % solution of formalin stabilised in phosphate buffer. The brain was removed, and great care was taken when removing the cannula from the brain, to try and minimise tissue damage. Brain was put into 10% formalin solution for 24 hours before being moved into a 30% W/V sucrose solution for at least 48 hours. Brains were sectioned using a microtome (40 µm coronal sections) before being mounted on slides and stained using cresyl-violet. Slides were viewed under a microscope and the cannula placement was noted and transferred to a drawing containing an outline of the marmoset brain.

Histological analyses by sectioning and staining using cresyl-violet has not yet been possible for subject 2 due to the impact of the ongoing human COVID-19 pandemic lockdown.

3. Effects of inactivation of the vlPFC on performance of variable and fixed self-ordered response sequences

3.1. Introduction

There is clear evidence supporting the involvement of the prefrontal cortex in self-ordered response sequencing in both humans (Owen et al., 1996) and non-human primates (Collins et al., 1998) (please see general introduction for more detail). A landmark study on the subject was published in the late 1990s in marmosets (Collins et al., 1998). In this study common marmosets with excitotoxic lesions extending over the lateral and orbital areas of the frontal cortex, as well as marmosets with extensive prefrontal depletion of monoamines, were investigated on a spatial self-ordered search task. In this task animals needed to respond to between 2 and 5 concurrently presented, identical, stimuli on a touch screen without repeating an already made response. Animals with excitotoxic lesions showed a very strong impairment in generating spatial self-ordered sequences, in contrast with monoaminergic depletion, which did not impair task performance. The impairment was perseverative in nature, animals showed a strong tendency to repeat the preceding response. A behavioural probe was utilised to remove the opportunity for continuous perseverative errors by hiding the previously selected stimulus until a new response had been made. The probe recovered performance and it was no longer significantly different from pre-operative baseline. To investigate whether delay-dependent working memory deficits could be responsible for the impairment, a further behavioural manipulation was performed where superimposed cues indicated previous responses. This behavioural manipulation did not rescue performance, animals still showed a perseverative impairment. A delay-dependent working memory impairment would have been expected in the monoamine depleted group, so a spatial delayed response task was also trained. In this task both the animals with excitotoxic lesions and the monoamine depleted group showed deficits. The findings indicate that the impairment in response sequencing following extensive prefrontal lesions is not due to an impairment in maintaining information online in working memory, but more likely an inability

to inhibit perseverative responding to the already performed responses (Collins et al., 1998). A follow up study was undertaken to understand which region of the PFC was responsible for the impairment. Excitotoxic lesions of the vlPFC, but not the OFC were shown to create a similar perseverative phenotype on the spatial self-ordered sequencing task (Walker et al., 2009a).

This raises questions regarding the precise contribution of vlPFC in organising sequential behaviour. vlPFC has been implicated in having a role in many different cognitive tasks. A study in rhesus macaques with ablations of ventral and orbital PFC used a conditional visuomotor learning task with varying attentional demand, concluded that vlPFC plays a role, not only in identifying stimuli of behavioural relevance, but also utilising that information to guide choice and action (Rushworth, 2005). Interestingly, the same ablation in a similar task showed a deficit in applying behavioural strategies, greatly impeding rapid learning of the task (Bussey et al., 2001). Selective ablations of the vlPFC, as opposed to the dlPFC and OFC, impair implementation of a successful reward-maximising strategy in a sequencing task by disorganising behaviour and causing a failure to implement rules (Baxter et al., 2008). So, it seems that in the performance of response sequences the role of vlPFC might be to enable the organization of behaviour when there is a demand for responding to be flexible and goal directed. However, the ablation technique used in these latter studies in the macaque are known to cause damage to fibres of passage and thus the precise region in which damage induced the response deficit is unknown (see e.g., Rudebeck and Murray, 2011).

In this chapter, data are presented that elucidate the contribution of vlPFC on performance of spatially determined sequences of responses using a technique in which only the target region is directly affected. Marmosets were trained to perform different sequencing tasks and had permanent indwelling cannulas implanted to allow infusions of drugs selectively into the vlPFC. Area 47/12 was inactivated using a combination of GABA-receptor agonists muscimol ($GABA_A$) and baclofen ($GABA_B$) and the effect it had on task performance was measured. Utilising cannulation procedures for temporary lesions, rather than permanent lesions offers a number of advantages. For example it allows a within-subject design, whereby animals act as their own controls and also enables different manipulations to be tested in the same animal. It also avoids any processes of recovery and compensation that can affect the interpretation of permanent lesion studies.

Specifically, four different self-ordered sequencing tasks were used in two experiments. The tasks required varying degrees of flexibility, **Table 3.1** shows an overview of the differences in features of the tasks and **Fig. 3.1** shows an overview of the variable tasks.

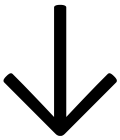
Flexibility requirement	Experiment	Task Name	Varying Difficulty	Errors punished	Variable sequences	Fixed sequence
	1	Variable 4-Block - Probe	X		X	
	1	Variable 4-Block	X	X	X	
	2	Variable 1-Block		X	X	
	2	Fixed 1-Block		X		X

Table 3.1 Task features. The table presents the elements that differ among the 4 tasks used in the two experiments of this chapter. Tasks are presented in a descending order of 'flexibility'. An X indicates that the feature is present in the task. Varying difficulty indicates that number of circles and vanishing time differ during the task. Errors punished indicates if an error causes trial abortion. Variable sequences indicate that animals perform a randomly generated sequence each trial, while fixed sequence is performance of the same sequence over multiple days.

The tasks used in this chapter were all based on an abstract rule – "Select each identical stimulus once and once only". Once an animal made a response, the responded-to stimulus disappeared for a short duration, the vanishing time (vt), before returning. Animals could continue responding to the other stimuli which were still 'active' during that time. This is in contrast to previous studies (Collins et al., 1998; Walker et al., 2009a) where there was a delay component involved, once a response was made, all the stimuli vanished, before all stimuli re-appeared concurrently. Two different perseverative error-types (Sandson and Albert, 1984) were possible with the present task design, categorised as continuous and recurrent perseverative errors. If, on a given trial, animals could respond to circles in position 1, 2 and 3, a continuous perseverative response sequence could be 1-1 or 1-2-2, whereas a recurrent perseverative error could only be the response sequence of 1-2-1. There are no other error types.

In experiment 1, two flexible 4 block sequencing tasks were used. They were identical in their design, with the difference that in the probe version, errors were not punished. The tasks were divided into 4 blocks of trials with three different difficulties. For each trial in these tasks, the sequence presented was randomly generated based on an 8-position grid (**Fig. 3.1 C**),

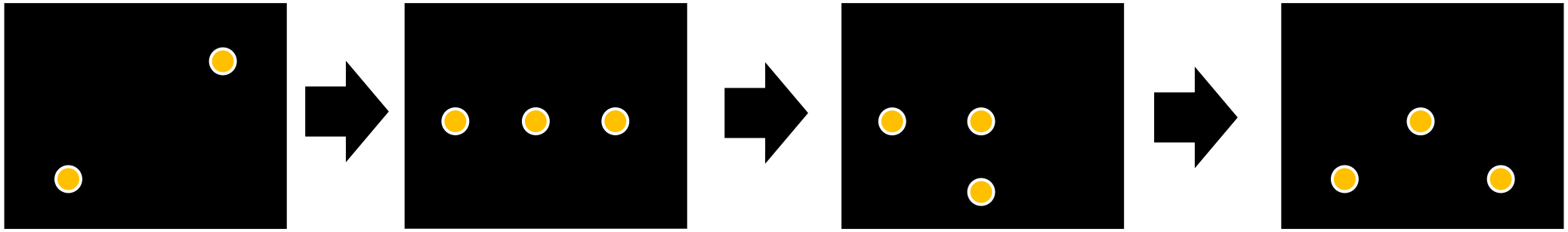
which means animals were presented with 56 different sequences. In the probe version of the task, animals were not punished for an erroneous response and could continue responding until all three responses had been selected. Responding was not punished per se, but superfluous responding increased the effort for reward, so animals needed to monitor their actions to reduce redundant responding.

In experiment 2, the effect of inactivation on a variable sequence task was contrasted with a fixed sequence task. To make experience comparable across experiments animals had the same pre-operative training but performed a simplified version of the variable task consisting of 3-circle trials of one difficulty, before being moved on to a fixed sequencing task. The fixed sequence performed was still derived from the same grid but the set of stimuli from that grid were fixed across trials and sessions. The task was still self-ordered. There was thus no longer a need to flexibly respond to a unique sequence each trial and animals could either heuristically learn a strategy to perform the fixed sequence in a relatively stereotyped manner or could still exhibit a degree of sequence variability if they chose to do so.

The hypothesis was that vlPFC would be required for successful performance of the variable sequencing tasks, but to a different degree. For tasks requiring greater flexibility, there would be a stronger dependence on the integrity of vlPFC functioning and hence a stronger impairment following inactivation. In the fixed sequence task, where the same sequence was learnt heuristically over days, the hypothesis was that the vlPFC, in contrast to the variable sequence tasks, would not be required for successful performance.

To summarise, the aim of the two experiments presented in this chapter was to determine how the vlPFC contributes to performance of response sequences by using local temporary inactivation of the area. Consequently, animals were tested on different sequencing tasks without a delay element and with a hierarchy of flexibility between tasks. In experiment 1 animals were trained to perform, in a flexible manner, variable sequences in blocks of alternating difficulty. Animals were also tested on a probe version of the task where erroneous responding was not punished, requiring animals to correctly track their actions and suppress superfluous responses. In experiment 2, animals were trained to perform a similar, but simpler, variable sequencing task followed by a fixed sequencing task with the goal to contrast inactivation of vlPFC on variable and fixed sequences.

A) 4-Block variable spatial self-ordered sequencing task (experiment 1)



Block 1: 10-16 2-circle trials. Vanishing time 0.5s

Circle positions randomised each trial

Block 2: 10-16 3-circle trials. Vanishing time 1s

Circle positions randomised each trial

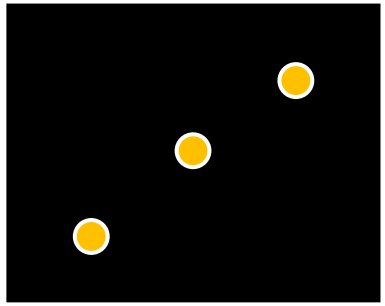
Block 3: 10-16 3-circle trials. Vanishing time 0.5s

Circle positions randomised each trial

Block 4: 10-16 3-circle trials. Vanishing time 1s

Circle positions randomised each trial

B) 1-Block variable spatial self-ordered sequencing task (experiment 2)

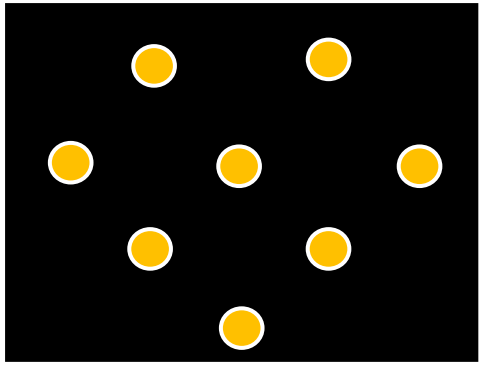


30 3-circle trials.

Vanishing time 0.5s

Circle positions randomised each trial

C) 8-position grid



The 8-position grid used to randomise circle positions

Figure 3.1 Variable spatial self-ordered sequencing tasks. A) Illustrates the 4-block spatial self-ordered sequencing task, the numbers of trials are the same for each block, but the number of trials within a block differ between subjects. After a stimulus is selected, it disappeared for the vanishing time, until it re-appears. Animals could continue responding throughout this time. B) Illustrates the 1-block spatial self-ordered sequencing task. All animals run 30 trials with a vanishing time of 0.5 seconds. C) 8-position grid used to randomise stimuli location for trials. 56 different sequences were generated from the 8 positions.

3.2. Methods

3.2.1. General methodology

3.2.1.1. Surgical procedures

After animals had received pre-operative training, they had cannulas implanted to allow for central infusion of drugs into the vlPFC, see **Fig. 3.2**. For more details on surgery, please see General Methods, Table 3.2. Subject 1 had the cannula implanted at an angle of 8°, subject 2 and 3 had the cannulas implanted at an angle of 10°, remaining subjects 4, 5, 6, 7 and 8 had their cannulas implanted at an angle of 9°. The angle was adjusted after histological analysis of animals in other studies. Subjects 6, 7 and 8 also had cannulas targeting the caudate, but no caudate data are presented in this chapter.

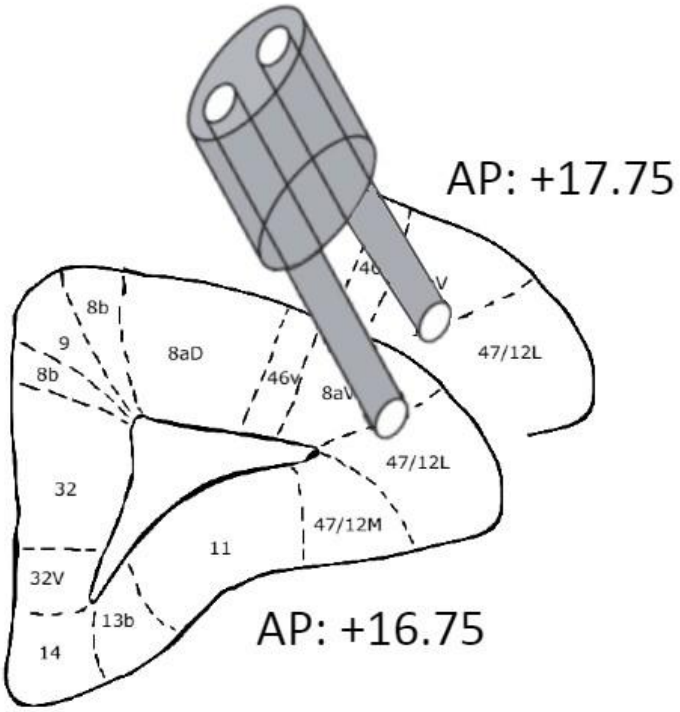


Figure 3.2 Schematic illustration of vlPFC cannulations. Image not to scale but illustrate the implanted double cannula, used to target the vlPFC at AP +16.75 and +17.75. Images only illustrate one hemisphere, but cannula were bilateral. For infusions an injector protruding 0.5 mm from the cannula was used. Part of image adapted from Clarke et al. (2015), brodmann area subdivisions from Paxinos et al. (2012).

3.2.1.2. Drug preparation and treatment

All animals in this study had a combination of muscimol ($GABA_A$-receptor agonist) and baclofen ($GABA_B$-receptor agonist) solution infused into the vlPFC to allow temporary inactivation of the area. **Table 3.2** shows number of testing sessions before first infusion. This muscimol and baclofen 'cocktail' (musbac) was made up in saline to a concentration of 0.1 mM muscimol and 1mM baclofen before being filtered and aliquoted. Aliquots were stored at -20° C for a maximum of 3 months. Musbac was thawed immediately before infusion. Fresh sterile saline was used for the control vehicle infusion.

The infusion was at a rate of 0.5µl per minute for one minute. A 25-minute pre-treatment time was allowed after infusion before testing. All animals had an injector protruding +0.5mm from the cannula to allow for infusion at 0.7mm from the base of the brain.

Subject	1	2	3	4	5	6	7	8
Sessions until first infusion	304	220	259	103	183	126	116	194

Table 3.2 Testing sessions before first infusion. Table shows the number of testing sessions animals performed, pre-operative and post-operatively, before reaching a stable responding on the task before having their first infusion. Subject 1 had a comparably high number due to task piloting before first infusion.

3.2.2. Experiment 1

3.2.2.1. Subjects

Five marmosets took part in experiment 1 (3 male, 2 female), for more details please see chapter 2, **Table 2.1**.

3.2.2.2. 4-Block variable spatial self-ordered sequencing task

All subjects in Experiment 1 run two versions of the 4-block variable sequencing task, a non-probe and a probe version. All blocks consisted of the same number of trials, but this varied between animals, from 10 to 16. Subject 1 had 16 trials per block, subject 4 had 14 trials per block, subject 3 had 12 blocks per trial, subject 2 and 5 had 10 trials per block. The inconsistency in trials is due to variability in motivation between animals. Animals started on

16 trials per block, but it was gradually reduced until animals performed all trials on a stable basis. Block 1 consisted of 2 circle trials with a vanishing time of 0.5s. Block 2 and 4 were identical, 3 circle trials with a vanishing time of 1s. Block 3, the most difficult block, consisted of 3 circle trials with a vanishing time of 0.5s. The reason for the addition of a block identical to block 2 after block 3 was to control for motivational aspects that might otherwise play a contributing role in any performance decline observed on block 3 after a manipulation, given that block 3 is towards the end of the task. In the non-probe task, an erroneous response caused trial abortion. In the probe session, animals were not punished for errors, but allowed to continue responding until they had selected each individual stimulus. Animals were required to monitor their responding for redundant responses and inhibit them accordingly. The probe task also enabled further investigation of errors. For the probe trial reward was provided, even if the trial was incorrect, if all three spatial locations were responded to. Before having any manipulations on the probe task, animals were habituated to the probe session on at least two separate sessions.

This task design allows for comparison with previous excitotoxic lesion studies that showed an impairment for both 2 and 3 circle trials (Walker et al., 2009a). The 4-block task provides insight into the role of vlPFC in performance of sequences with changing difficulty. The number of circles change, but also vanishing times. This requires subjects to adapt to the number of circles presented, but also adapt attentional requirements when the vanishing time changes. As presented in the introduction, vlPFC has been implicated in performance of a number of cognitive tasks, which all require behavior to be flexible. It is thus of interest to investigate the effect of inactivation in a task with alternating instead of constant task demands. During the non-probe task trials were aborted if an error was performed and a new sequence was presented instead. The non-probe task does therefore not provide any information about the ability of animals to rescue performance after an error. The probe version of the 4-block task was designed to investigate error correction or perseveration. It has been suggested that maladaptive, lPFC dependent, behavioural planning could lead to perseveration (Genovesio et al., 2006). Previous excitotoxic studies have demonstrated that after lesions to the vlPFC, animals perseverate by being unable to inhibit superfluous erroneous responses (Walker et al., 2009a).

3.2.2.3. Data analysis

This experiment used a within-subject design. Testing data were collected in a Microsoft access database. Data were exported into Microsoft Excel (Office 365) and R studio (Version 1.2.1335, RStudio: Integrated Development for R. RStudio, Inc., Boston, MA). Data were transferred from RStudio and Excel before statistical analysis was run in GraphPad Prism (Version 7.03 for Windows, GraphPad Software, La Jolla, California, USA).

Data were presented as mean values with the standard error of the mean (SEM). A two-way repeated measures analysis of variance (ANOVA) was performed. Post-hoc tests were corrected for using Sidak's correction for multiple comparison. $P < 0.05$ was used for statistical significance and denoted with *, $P < 0.01$ was denotes as **.

3.2.3. Experiment 2

3.2.3.1. Subjects

Three marmosets took part in experiment 2 (2 female, 1 male), for more details please see Chapter 2, **Table 2.1**

3.2.3.2. 1-Block variable spatial self-ordered sequencing task

The animals in experiment 2 ran a simplified version of the 4-block variable spatial self-ordered sequencing task (see **3.2.2.2**). Consisting only of 1 block with 30, three circle trials with a vanishing time of 0.5s. Errors were punished by trial abortion.

3.2.3.3. Fixed self-ordered sequencing task

After completing manipulations on the 1-block variable sequencing task subjects were moved onto a fixed version of the task. This task was designed to allow investigation of the same manipulations on a similar task but with fixed sequences. In this task animals still performed, in a self-ordered manner, 3 circle trials with a vanishing time of 0.5s, but with the same spatial sequence of three responses every session for at least ten days before manipulations.

The sequence used was different for each subject and based on data from the flexible spatial self-ordered sequencing task from the previous two months of testing; prior to starting the fixed sequencing task. To make sure the fixed sequences was not already performed in a rigid fashion, and to allow responding to improve without reaching an immediate ceiling effect; the fixed sequence was based on three criteria: First, the subject had to have made at least 5

of the 6 possible correct response sequences during these two months. Second, there was not to be a strong bias towards a specific correct response in the sequence; the percentage of trials for which each correct response was performed was approximately equal. Third, animals needed to have an accuracy score on the sequence of around 50%, which was much superior than chance (21%), but still with room for improvement. Animals performed the task for at least ten days to allow for possible strategies to develop before they had any infusions on the task.

3.2.3.4. Data analysis

Experiment used a within-subject design. Testing data were collected in a Microsoft access database. Data were exported into Microsoft Excel (Office 365) and R studio (Version 1.2.1335, RStudio: Integrated Development for R. RStudio, Inc., Boston, MA). Data were transferred from RStudio and Excel before statistical analysis was run in GraphPad Prism (Version 7.03 for Windows, GraphPad Software, La Jolla, California, USA).

A two-tailed paired t-test was performed. Data were presented as mean values with the standard error of the mean. $P < 0.05$ was used for statistical significance and denoted with *, $P < 0.01$ was denotes as **.

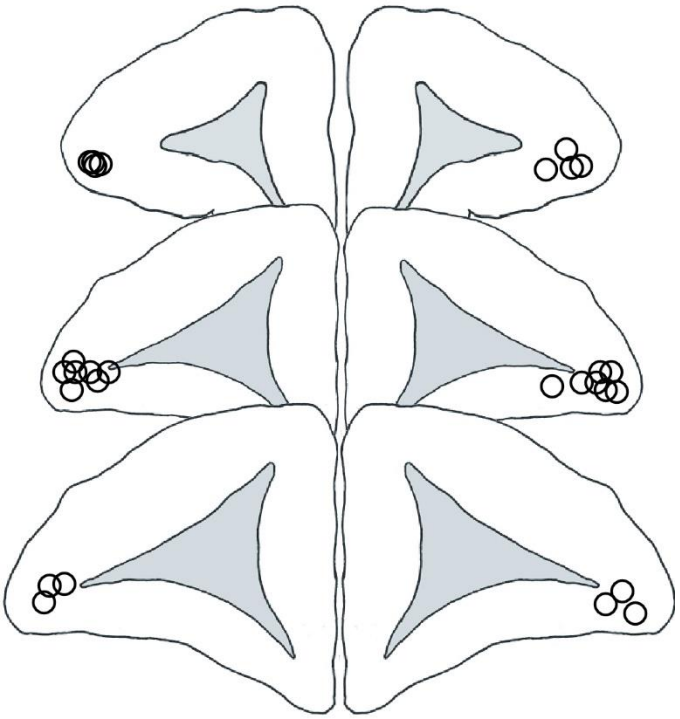


Figure 3.3 Cannula placements. Image illustrate end of cannula guides where complete or preliminary histological analysis have been performed Image adapted from Clarke et al (2015).

3.3. Results

3.3.1. Histology

Histological analysis of cannula placements revealed that all animals had cannulae successfully implanted targeting vlPFC 47/12, see **Fig. 3.3**. However, subject 2 is still pending histological analysis.

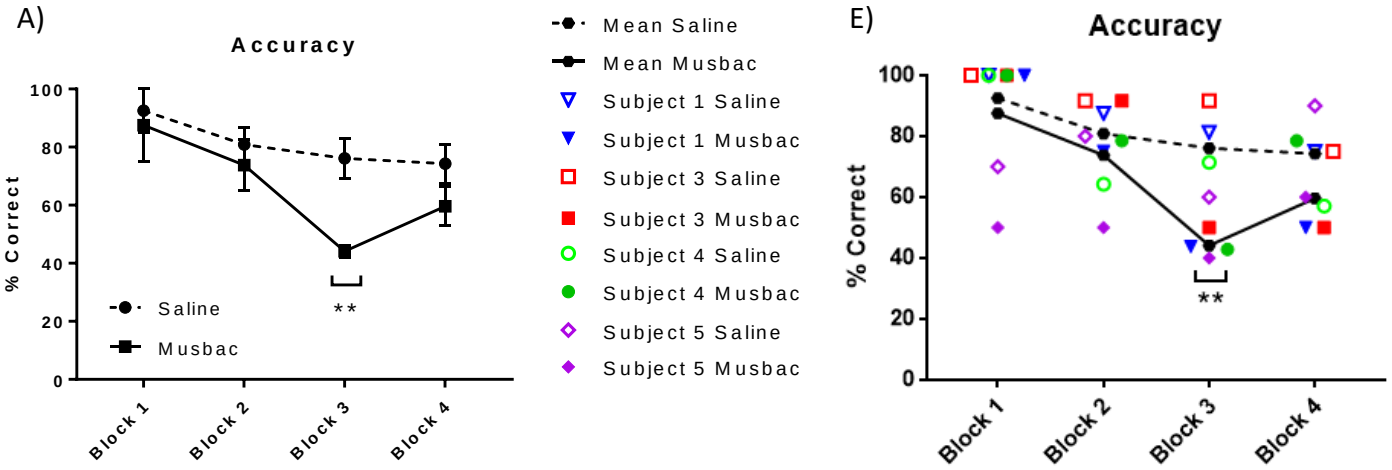
3.3.2. Experiment 1

3.3.2.1. 4-Block flexible spatial self-ordered sequencing task

The 4-block non-probe task was designed to investigate performance of both 2 and 3 stimuli spatial sequences with alternating task demands. **Fig. 3.4** shows that Inactivation of the vlPFC using musbac impaired task performance in the critical third and most difficult block of the task, by increasing the number of recurrent perseverative errors.

A two-way repeated measures ANOVA of accuracy showed a significant interaction between block and treatment (musbac) ($F(3,9)=3.895$, $p= 0.049$), post-hoc testing revealed that the only significant effect was for block 3 ($p=0.006$) (**Fig. 3.4 A,E**). Musbac did not affect total trials completed ($F(1,3)= 0.009$, $p= 0.931$) (**Fig. 3.4 B,F**).

To understand the nature of the sequencing deficit, continuous and recurrent perseverative errors per trial were investigated separately. Musbac did not affect continuous perseverative errors (Main effect: $F(1,3)=1.295$, $p=0.3378$. Interaction with block: $F(3,9)=1.074$, $p= 0.408$) (**Fig. 3.4 D,H**). However, for recurrent perseverative errors, whilst there was no main effect of musbac ($F(1,3)=4.824$, $p=0.116$)), there was an interaction between block and treatment ($F(2,6)=5.761$, $p=0.040$), *post hoc* testing revealed a significant increase in recurrent perseverative errors only in block 3 ($p=0.014$) (**Fig. 3.4 C,G)**.



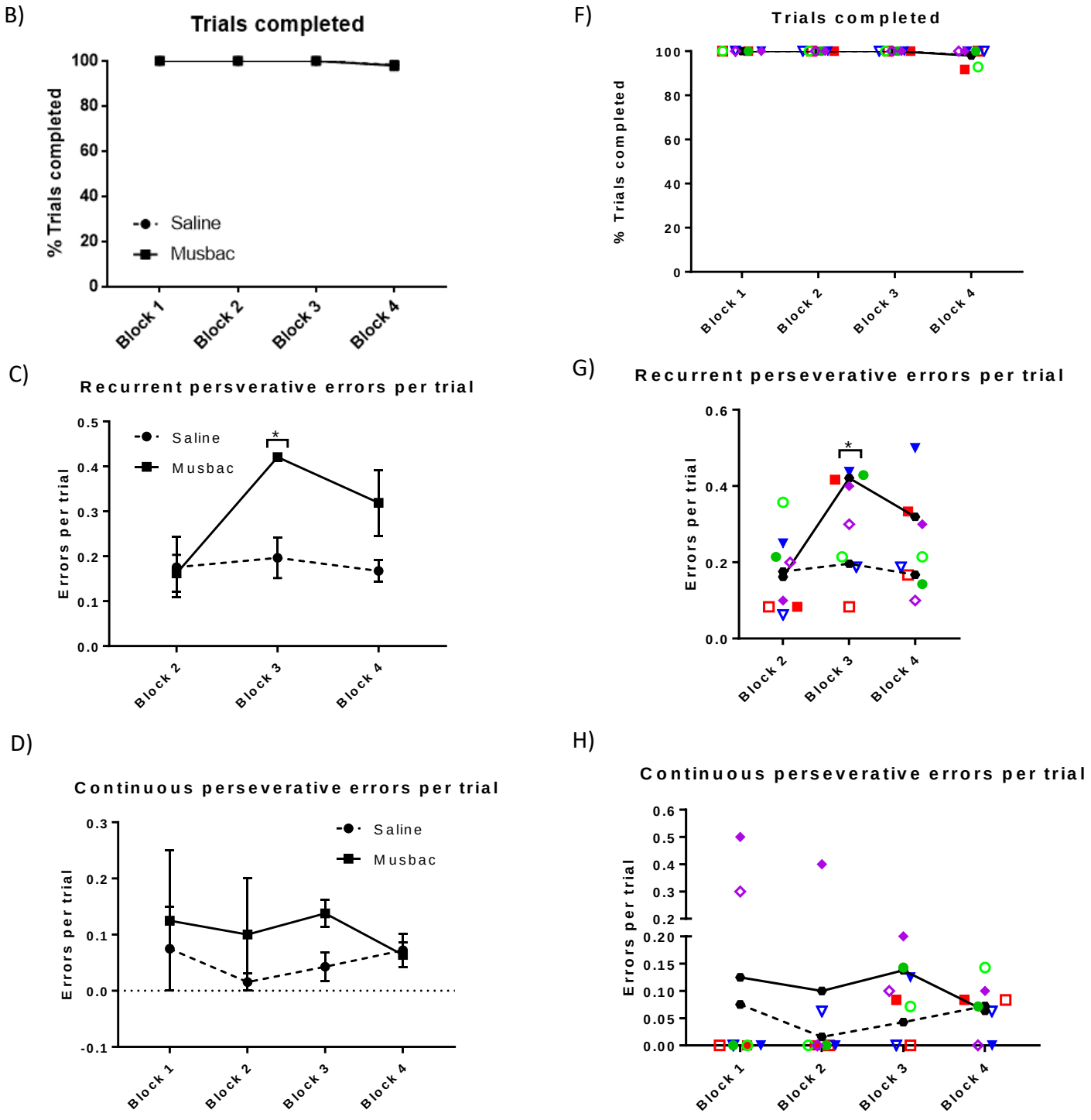


Figure 3.4 Performance measures on the 4-block flexible spatial self-ordered sequencing task. All graphs depict average data for the entire block. Graphs A-D shows mean values with standard error. If errors bars are shorter than the symbol, they are omitted. Graphs E-H show individual data points without error bars. A,E) Accuracy measured as correct trials, there is a highly significant effect of treatment on block 3 (p=0.006). B,F) Trials completed is all trials on which animals did not fail to respond, whether correctly or incorrectly. C,G) Average recurrent perseverative errors per incorrect and correct trials. The effect of treatment on recurrent perseverative trials was statistically significant in block three, denoted by a star. D,H) Average continuous perseverative errors per correct and incorrect trials.

Five different latencies were also investigated:

- Trial time; from stimulus presentation to completed sequence or trial error.
- Initiation time; from stimulus presentation to first response.
- Performance time; from first to last stimulus selection.
- Inter-response times; from first to second and second to third stimulus selection.

Inactivation of the vlPFC produced a trend towards slowed median performance time, see **Fig. 3.5** probably driven by the significant effect of a slowed inter-response time between 1 and 2 stimulus selections across all blocks, see **Fig. 3.6**.

There was no main effect of treatment on median trial time ($F(1,3)=1.228$, $p=0.349$) (**Fig. 3.5 A,D**), initiation time ($F(1,3)=0.863$, $p=0.421$) (**Fig. 3.5 C,F**), or inter-response time 2-3 ($F(1,3)=1.352$, $p=0.3290$) (**Fig. 3.6 C,D**). There was however a strong trend for musbac to slow median performance time ($F(1,3)= 9.336$, $p=0.055$) (**Fig. 3.5 B,E**) and there was a significant effect of musbac on median time from inter-response 1-2 ($F(1,3)=10.21$, $p=0.049$) (**Fig. 3.6 A,B**). The main effect of treatment on increased median latency on inter-response 1-2 was not significant for correct trials ($F(1,3)=4.42$, $p=0.126$) or incorrect trials separately ($F(1,3)=0.119$, $p=0.752$).

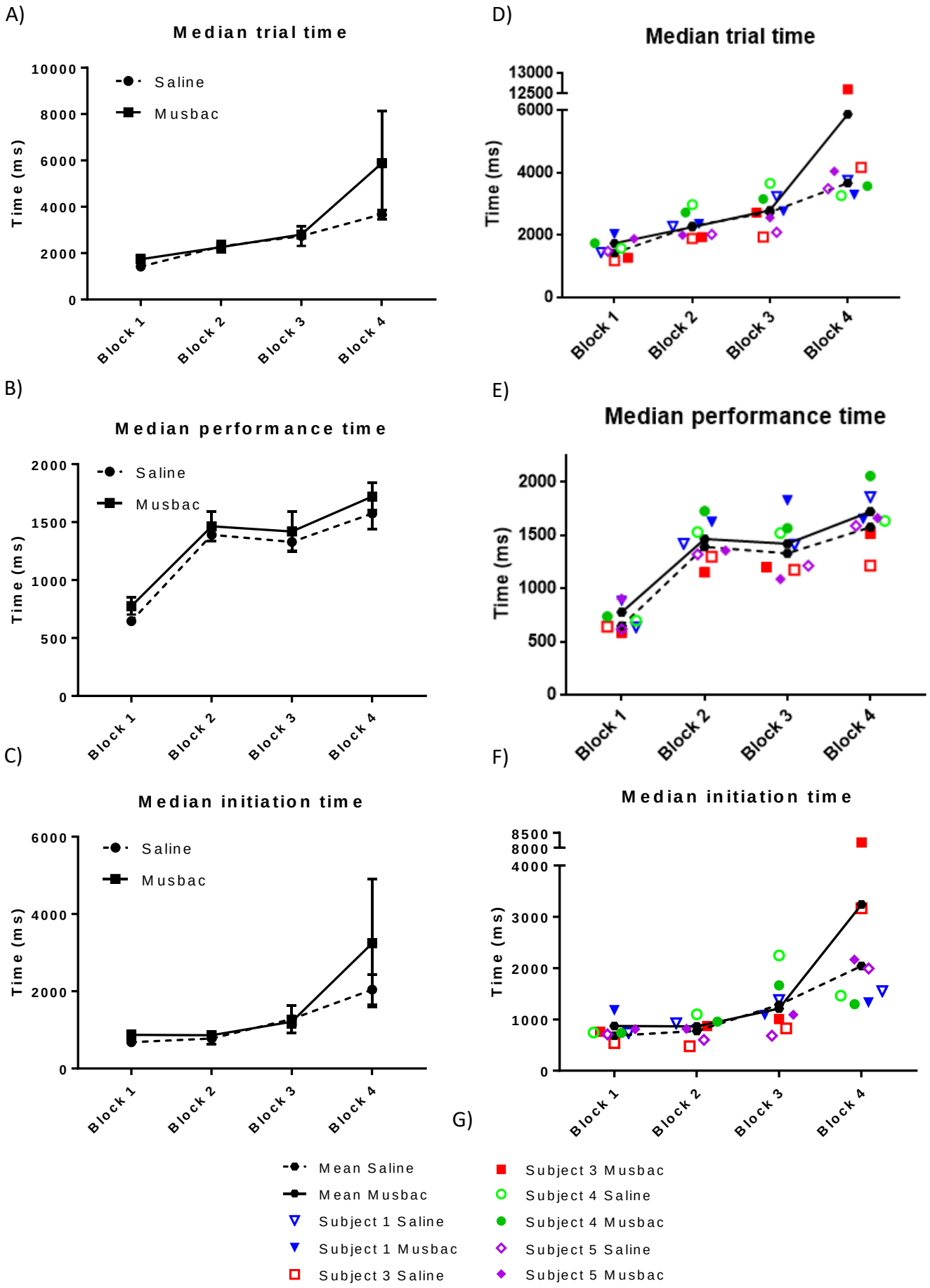


Figure 3.5 Latency data for the 4-block flexible spatial self-ordered sequencing task All graphs depict median data for the block. Graphs A-C shows median values with standard error. If errors bars are shorter than the symbol, they are omitted. Graphs D-F shows individual data points without error bars. A,D) Trial time indicates the time from presentation of stimulus to reward. B,E) Performance time is from first to last response. C,F) Initiation time is from stimulus presentation until first response.

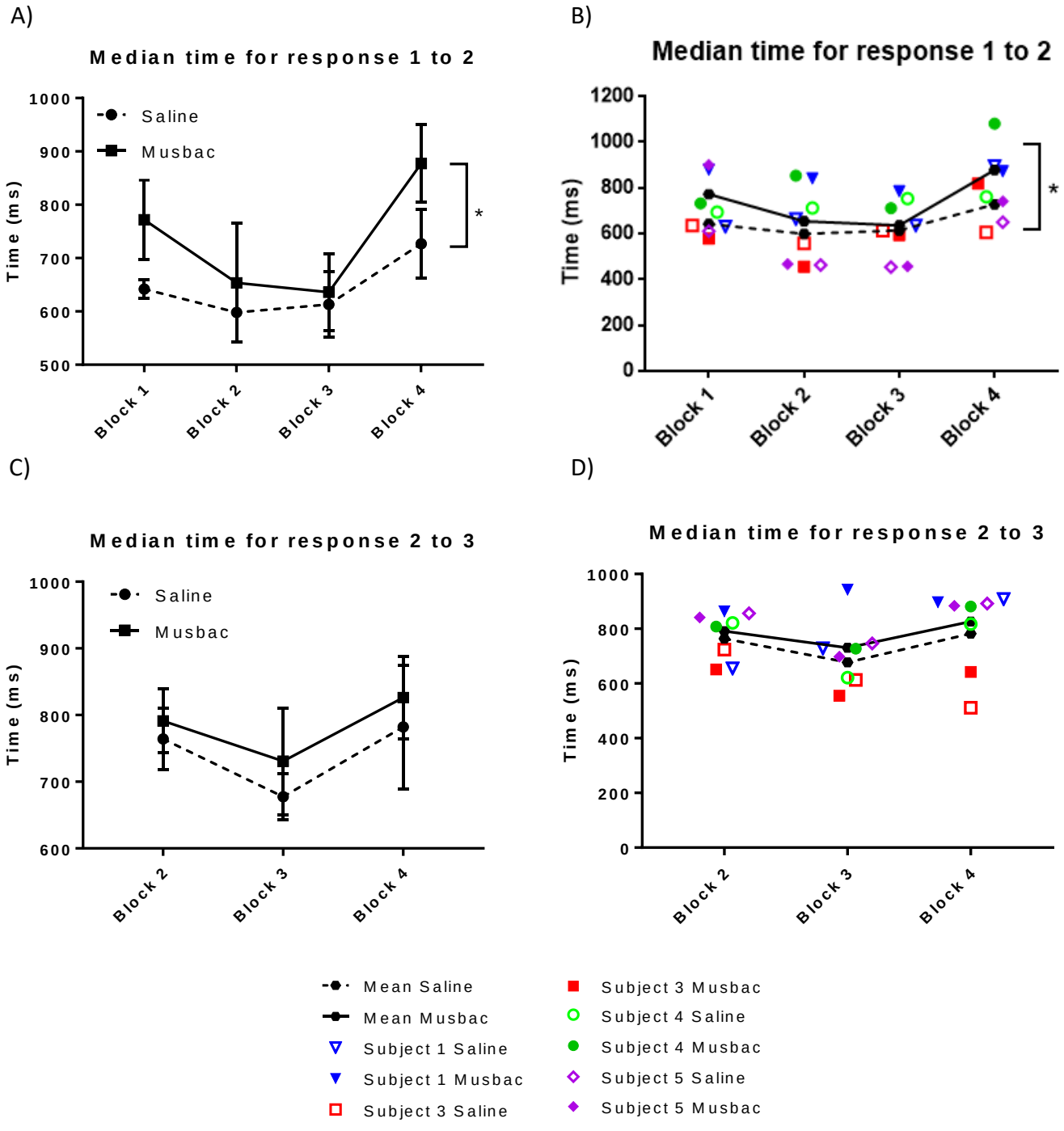


Figure 3.6 Latency data for inter-response 1-2 and 2-3 in the 4-block flexible spatial self-ordered sequencing task All graphs depict median data for the block. Graphs A-B shows median values with standard error. If errors bars are shorter than the symbol, they are omitted. Graphs C-D shows individual data points without error bars. A,C) Time between first and second response, musbac slowed this measure (p=0.049). B,D) Time between second and third response.

3.3.2.2. 4-Block flexible self-ordered sequencing task – probe task

The probe version of the 4-block task was designed to investigate perseveration after an initial error on the 4-block task. In the probe task errors were not punished by trial abortion and subjects were thus required to supress additional erroneous responses and complete the sequence. **Fig. 3.7** shows that inactivation of the vlPFC with musbac impaired sequencing on all blocks of the probe task. It significantly reduced accuracy, increased the number of errors, as well as the number of errors made on erroneous trials. **Fig. 3.8** shows that inactivation significantly increased the number of recurrent perseverative errors, with only a trend towards increased continuous perseverative errors.

There was a main effect of musbac on accuracy ($F(1,4)=38.51$, $p=0.003$) (**Fig. 3.7 A,D**), inactivation of the vlPFC impairing task performance. Errors per trial were also increased ($F(1,4)=23.64$, $p=0.008$) (**Fig. 3.7 B,E**). Musbac also increased the number of errors made on erroneous trials ($F(1,4)=10.61$, $p=0.031$) (**Fig. 3.7 C,F**). Examining the different error-types separately, there was a main effect of treatment on recurrent perseverative errors per trial ($F(1,4)=8.49$, $p=0.044$) (**Fig. 3.8 A,C**), but not on continuous perseverative errors per trial ($F(1,4)=5.658$, $p=0.076$) (**Fig. 3.8 B,D**). Only one animal made an omission, subject 3 made one omission in the fourth block when treated with musbac. Musbac had no effect on completed trials ($F(1,4)=1$, $p=0.373$).

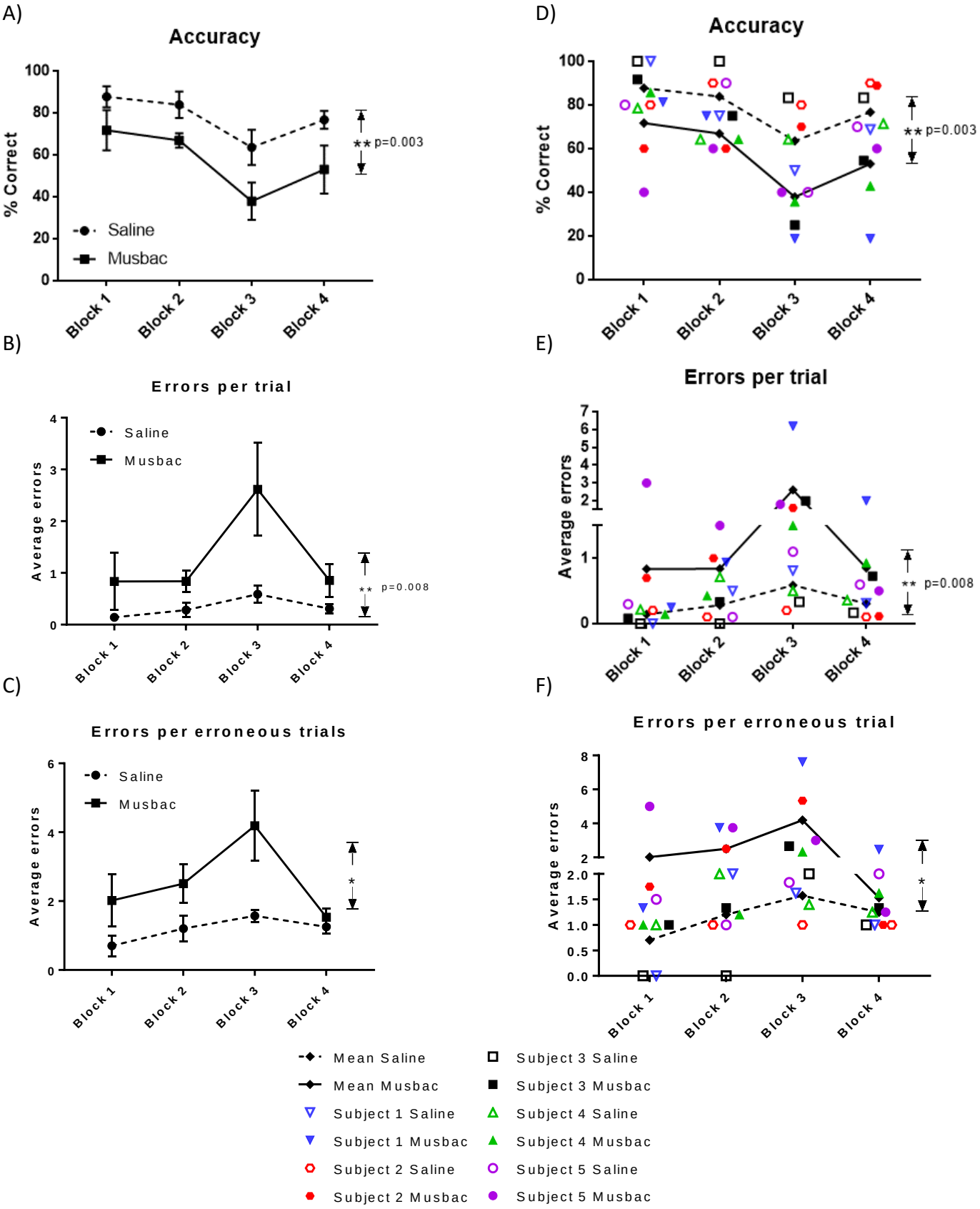


Figure 3.7 Performance measurements on the 4-block flexible spatial self-ordered sequencing probe task. All graphs depict average data for the entire block. Graphs A-C shows mean values with standard error. If errors bars are shorter than the symbol, they are omitted. Graphs D-F show individual data points without error bars. Two arrows with star(s) denote a significant main effect of treatment on the measure. A,D) Accuracy per block, a correct trial being when animals completed a three-response sequence without errors. B,E) Errors per trial denotes the number of errors made divided by the number of trials per block. C,F) Average number of errors made per block divided by incorrect trials in that block.

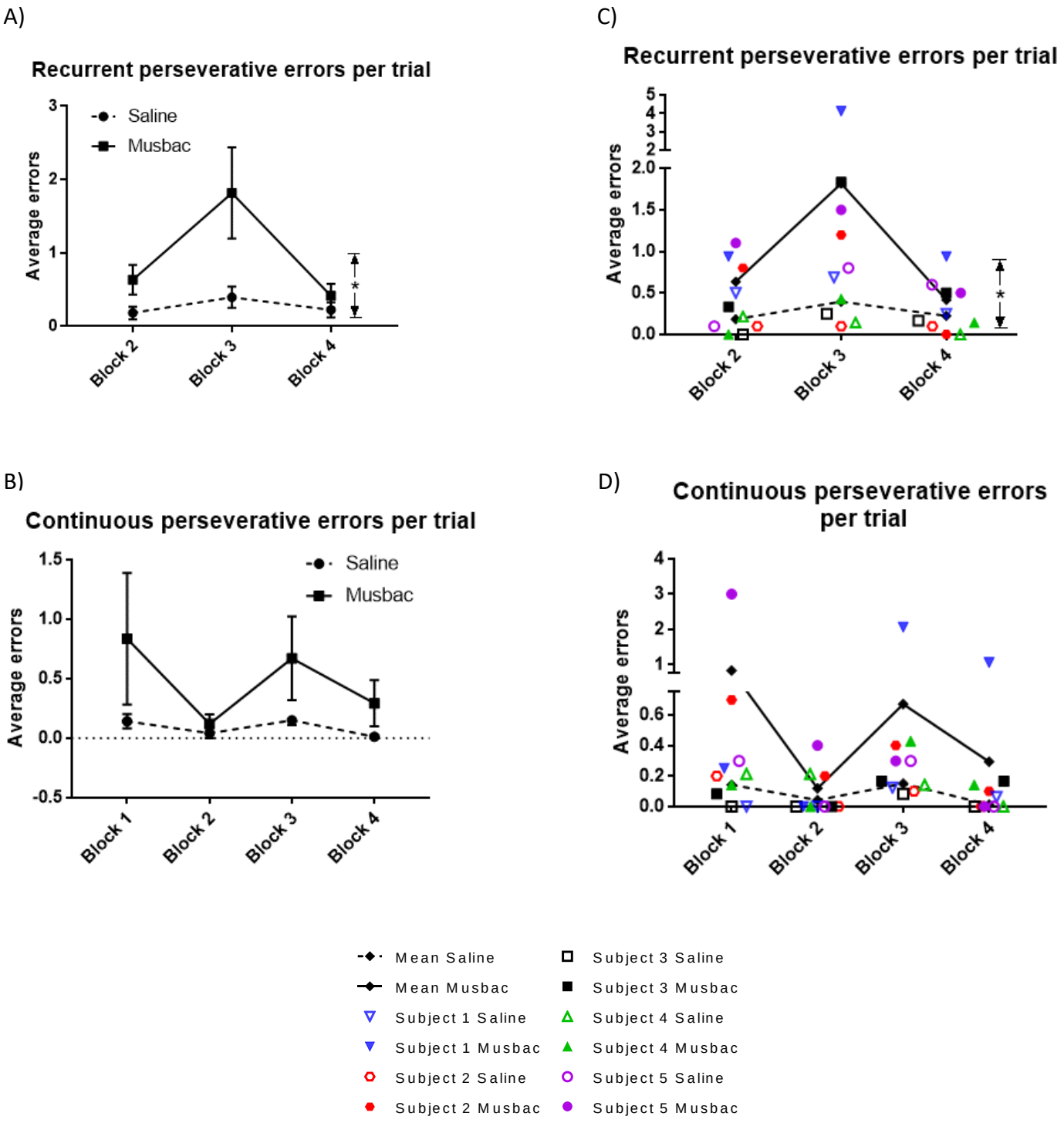


Figure 3.8 Individual error types on the 4-block flexible spatial self-ordered sequencing probe task. All graphs depict average data for the entire block. Graphs A,B shows mean values with standard error. If errors bars are shorter than the symbol, they are omitted. Graphs C,D shows individual data points without error bars. Two arrows with star denote a significant main effect of treatment on the measure. Number of recurrent perseverative errors (A,C) and continuous perseverative errors (B,D) made in a block divided by trials per block

3.3.3. Experiment 2:

3.3.3.1. 1-Block variable spatial self-ordered sequencing task

The 1-block task was designed to consist only of the trials showing a significant impairment in the non-probe version of the 4-block task previously presented in this chapter. **Fig. 3.9** shows that all three animals showed impaired performance of this revised task when vlPFC was inactivated using musbac.

A two-tailed paired Student's t-test showed that there was a significant effect between the groups on accuracy, mean ± SEM difference for Musbac – Saline = -16.67 ± 1.923, p=0.013 (**Fig. 3.9 A**).

All three animals showed a higher number of recurrent perseverative errors, (**Fig. 3.9 B**) but this effect was not significant with a two-tail paired t-test, mean ± SEM difference for Musbac – Saline = 4.667 ± 1.33, p=0.073. The same test was performed on continuous perseverative errors (**Fig. 3.9 C**) and there was no significant effect, mean ± SEM difference for Musbac – Saline = 0.33 ± 0.88, p=0.741. There were no omissions under any manipulation.

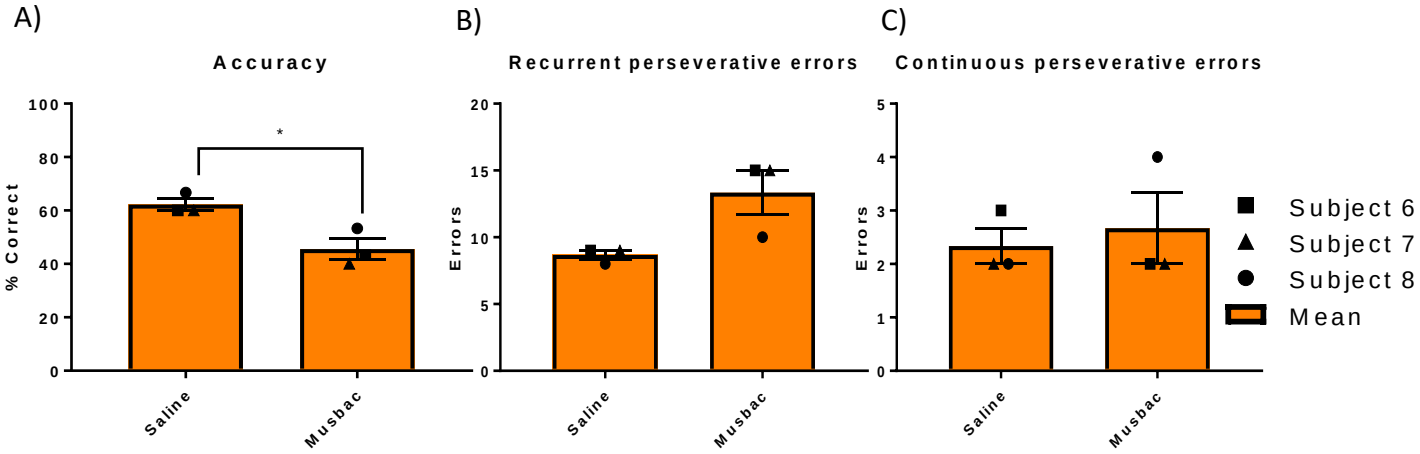


Figure 3.9. Performance of the 1-block variable spatial self-ordered sequencing task. The two bars represent the mean for the different manipulations, with individual data-points. Error bars represent standard errors. All graphs have the same key, presented on the far right. A) There was a significant main effect of treatment on accuracy. B) All animals made more recurrent perseverative errors upon inactivation of the vlPFC, but this effect was not statistically significant. C) There was no clear effect on continuous perseverative errors.

3.3.3.2. Fixed self-ordered sequencing task

Fig. 3.10 shows all the correct responses made during performance of the fixed task. As can be seen all animals on first presented with a fixed sequence showed a wide range of response sequences when solving the task, each colour representing an alternative correct sequence. In all cases this narrowed down somewhat across training so by the end they were performing fewer sequences. **Fig. 3.11** show that further breakdown of these correct responses indicates that all three animals adopted a strategy to solve the sequence, but only two out of three consistently performed it over many days. One animal, subject 6, adopted a strategy by solving the sequence through moving across the screen in a counterclockwise fashion. Another animal, subject 8, adopted a strategy where she almost exclusively started with the circle in position two, before selecting either of the other two circles. The last subject, subject 7, adapted a clockwise strategy for ten testing days before showing greater flexibility in his responding again. **Fig. 3.12** shows that for animals trained on a fixed, specific sequence, inactivating the vlPFC using musbac no longer impaired task performance. Inactivation using musbac was without effect on accuracy (**Fig. 3.12 A**); mean ± SEM difference for Musbac – Saline = 5.56 ± 4.008, p=0.299 (two-tailed paired t-test). Similarly, there was no effect on continuous perseverative errors (**Fig. 3.12 C**), mean ± SEM difference for Musbac – Saline = 1.33 ± 2.028, p=0.578 or recurrent perseverative errors (**Fig. 3.12 B**), mean ± SEM difference for Musbac – Saline = 0.33 ± 2.404, p=0.902.

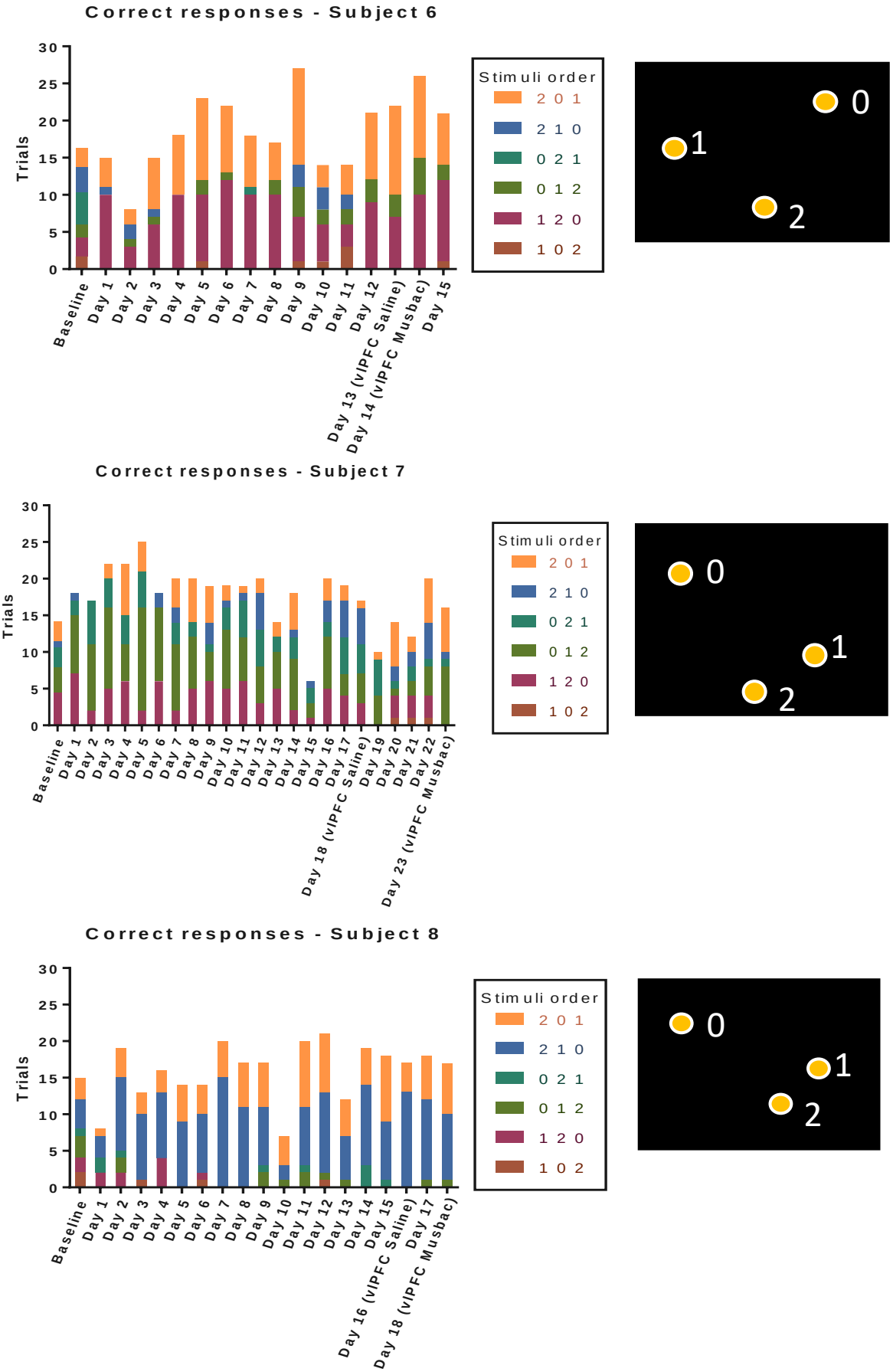


Figure 3.10 Correct responses on the fixed self-ordered sequencing task. Graph represents the correct responses made per session for each individual animal, the total height of the bar gives the accuracy while each colour on the bar represents a specific response sequence. The baseline data-point represents every time the specific sequence was performed over two months, before starting fixed sequence task. Baseline data points were converted to into a 30-trial representation. The black rectangle next to each graph illustrates the sequence performed by the subject corresponding to the graph. Numbers in the triangle indicate the number for each spatial position.

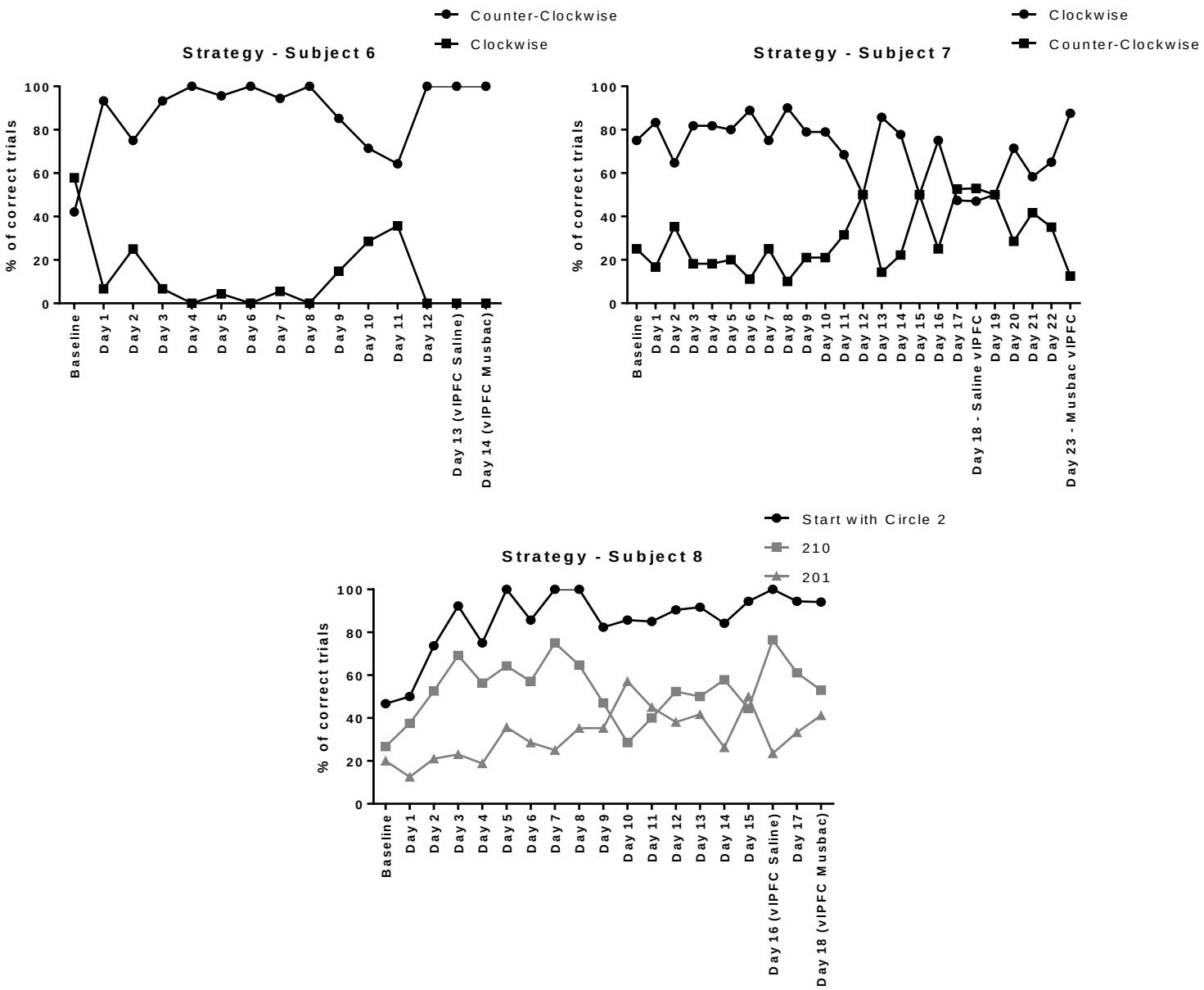


Figure 3.11 Strategy usage on the fixed self-ordered sequencing task. Correct responses on the fixed self-ordered sequencing task was grouped into strategies. The graphs illustrate how often a given strategy was performed on correct trials. The baseline data-point represents every time the specific sequence was performed over two months, before starting fixed sequence task. The grey data-points in the strategy graph for subject 8 represents the individual responses that make up the strategy.

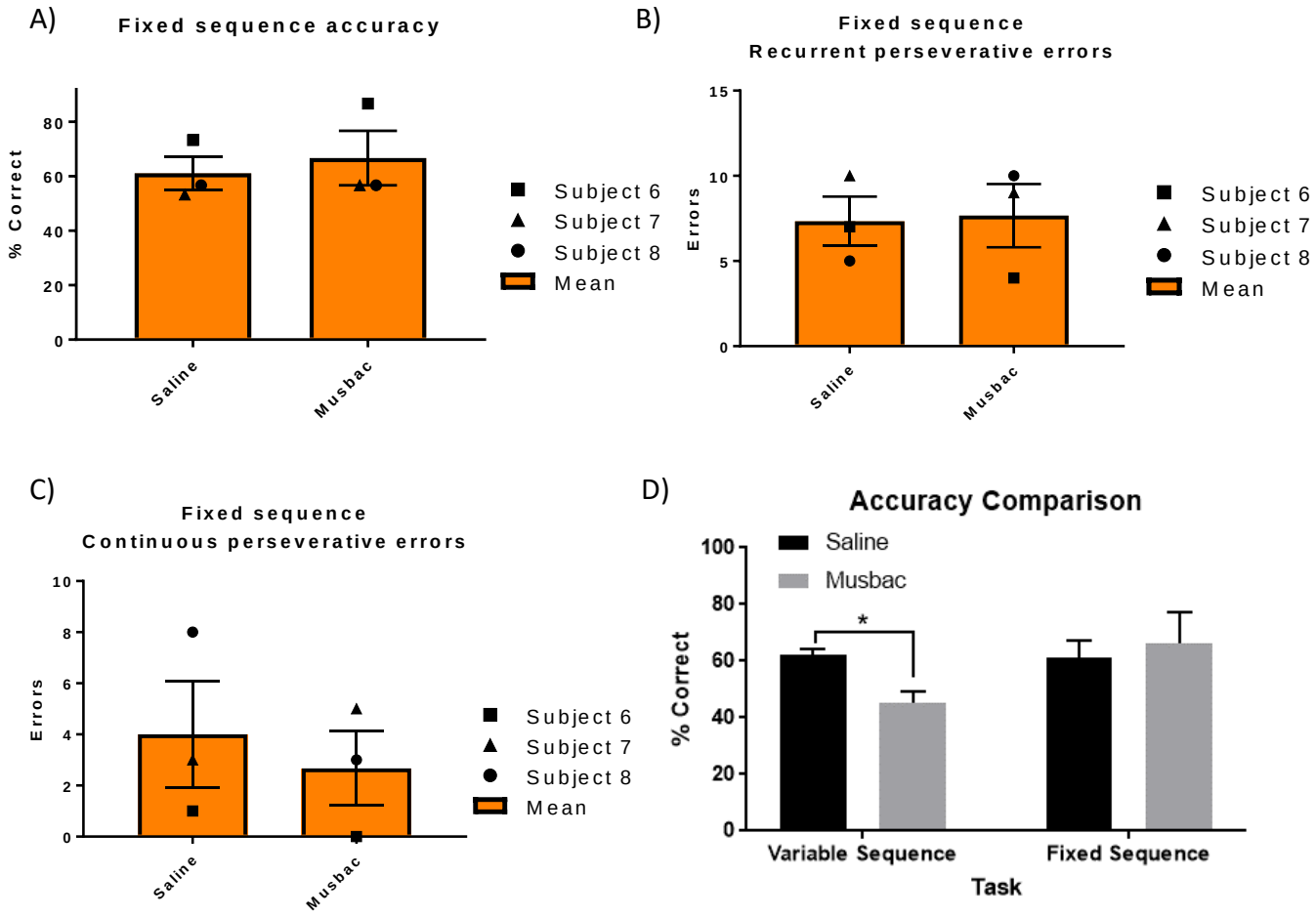


Figure 3.12. Performance scores on the fixed self-ordered sequencing task. A) Bar charts represent mean accuracy on task for both treatments, with individual data points. Error bars represent SEM. B,C) Bar charts depict mean recurrent perseverative (B) and continuous perseverative (C) errors under both conditions, with individual data points. Error bars represent SEM. D) A comparison of the task accuracy, with standard error, under saline and musbac for the variable **(3.3.3.1)** and fixed sequencing task. The impairment seen in the variable task disappears when animals perform fixed sequences.

3.4. Discussion

The data presented in this chapter confirm and extend previous findings in marmosets that show impairment of spatial self-ordered sequences after excitotoxic lesion of the vlPFC (Collins et al., 1998; Walker et al., 2009a). The data presented here show that the dependence on vlPFC function for successful performance remains even if the delay component of the task is removed. It also shows that tasks with higher requirements for flexibility had a stronger dependency on vlPFC function. However, once fixed sequences, as opposed to variable sequences, were performed, vlPFC inactivation no longer impaired task performance.

All four different sequencing tasks used in this chapter have subtle, but very important differences when trying to understand the nature of the impairment following inactivation of the vlPFC. In the most flexible sequencing task, animals performed 2 and 3 circle trials, with two different vanishing times on the 3 circle trials. Errors were also not punished by trial abortion, as in all other tasks. In this task, inactivating the vlPFC caused a significant decrease in accuracy across all blocks. The impairment was at two levels, at one level animals completed fewer correct sequences, but at a second level, animals were also impaired in suppressing additional superfluous responses having made an error. It could have been possible for animals to make fewer correct sequences as compared to saline, but not to have increased the overall number of errors on each incorrect trial. This indicates that not only is vlPFC responsible for planning successful self-ordered sequence behaviour, but also once the plan is lost by inactivation, animals were unable to adapt their performance to rescue excessive superfluous responding. In the standard 4-block task where errors were punished by trial abortion, inactivation of the task only impaired performance in the most difficult block. In the variable task in experiment 2, animals only performed the difficult trials from the 4-block tasks in experiment 1. These animals were also impaired, indicating that it is a sequencing deficit per se, and not simply an impairment in adapting to environments with alternating levels of difficulty.

In contrast to the above, vlPFC inactivation did not impair performance when animals were performing a self-ordered task but with the same three spatial locations on each trial. Here, animals performed this task over days and heuristically learned their own individual strategies to perform the sequence. This suggests that the vlPFC is not required for executing fixed

response sequences but, together with the significant deficits on the variable sequences, implies a role for the vlPFC in flexibly guiding behaviour in situations where a general principle needs to be applied to a larger set of problems.

A limitation of this study is that the order of the fixed and the variable sequencing task was not counter-balanced, all animals performed the variable sequencing task before the fixed sequencing task. It could be argued that the loss of effect following inactivation was due to tolerance developed to the drug or due to the learning of compensatory strategies, possibly during additional training occurring after the infusions. It seems unlikely that the loss of effect is due to drug tolerance. Animals in experiment 1, like animals in experiment 2, had two infusions of musbac (on the 4-block non-probe and probe tasks) and the drug showed an effect on both occasions whereas in experiment 2, musbac only showed a deficit on the first occasion. It is also unlikely that the difference is due to compensatory learning occurring on the variable task. The 3 animals that performed the 1-block variable and fixed sequencing task in experiment 2 had performed fewer testing sessions than the animals in experiment 1 by the time that infusions were initiated (see **Table 3.2**). Some animals in experiment 1 had performed over 100 more sessions than the animals in experiment 2 and yet still showed effects of inactivation on task performance. It is however a limitation of the study that needs to be acknowledged; future studies should counter-balance the tasks or perform a second infusion on the variable task after completing manipulations on the fixed task. This design was not followed because of pragmatic considerations; it would have taken too long to re-train marmosets on the variable task following fixed order testing. Also, having learned fixed order testing, learning to perform the more flexible tests may have proven very difficult indeed.

It could be argued that the impairment seen following temporary lesions to the vlPFC is an impairment in working memory, an inability for subjects to remember previous responses. However, it has been shown that lesions of area 47/12l, in rhesus macaques do not impair performance of a delayed match to sample task (Rushworth et al., 1997), indicating that vlPFC is not needed when information needs to be kept “online” during a delay. Efforts were made to decrease the working memory load of the tasks presented in this chapter, such that there was no delay element in the tasks. This contrasts with previous studies in the marmoset; but even without the delay element, the impairment persisted. In previous studies, a manipulation that cued previous responses did not rescue performance following lesions, but

one that made continuous perseveration impossible did however rescue performance (Collins et al., 1998). However, it should be pointed out that almost no task is without dependence on working memory, even though steps have been taken here to reduce it almost totally. By comparing the 4-block task in experiment 1, with a very similar task (but with a delay element) used in a previous study in the marmoset (Walker et al., 2009a), an impairment of almost the same size can be observed. In Walker et al. (2009) marmosets with excitotoxic lesions of the vlPFC showed around a 45% reduction in accuracy on 3-circle trials. In the 4-block task presented here animals showed an accuracy reduction of around 35% in the most difficult 3-circle task, following inactivation. The excitotoxic lesions were however larger and extended further anterior than the area that was targeted in this Chapter. There is however a qualitative difference between this study and the previous studies in the marmoset (Collins et al., 1998; Walker et al., 2009a). In this study the response impairment is, recurrent perseverative, as opposed to continuous perseverative in nature. This difference in error type is most likely attributed to differences in task design. In the current task, once a response has been made, it is impossible to make a continuous perseverative error during the vanishing time, so subjects continue responding during that time. As soon as the stimuli reappears, animals revisit it. On the other hand, in previous studies, animals were unable to respond to any stimulus during the post-response delay, meaning that for every response, animals are always presented with the option to revisit the stimulus (continuous error) or make a new response.

vlPFC has rich connections with visual association areas of the inferotemporal cortex (Roberts et al., 2007) and vlPFC has been shown to have a bias to stimulus encoding, rather than response encoding (Wallis and Miller, 2003). In tasks that allow separation of encoding object features and behavioural goals, it has been shown that vlPFC starts representing behavioural goals shortly after encoding object features (Yamagata et al., 2012). Many tasks that have shown an impairment in strategy following lesions to the vlPFC used elements of visual discrimination (Baxter et al., 2009; Bussey et al., 2001), where responses had to be guided to different visual stimuli.

A role for the vlPFC in flexible behaviour on visual extra- and intra-dimensional shifts has also been established. A 1996 study showed that lesions of the vlPFC impairs extra-dimensional shifts, while leaving performance of intra-dimensional shifts intact (Dias et al., 1996). This impairment has been extended to simpler one-dimensional shifts as it was shown that lesions

of vlPFC are without effect on a reversal learning task when a rule has already been learnt, but it does impair performance when novel stimuli are introduced (Rygula et al., 2010). There are similarities between the findings of Rygula (2010) and the findings presented here; once a rule has been trained and learnt, vlPFC task dependence is lost. The task differences are however very important for our understanding of vlPFC function. Due to its very rich connections to visual association areas, it could be argued that the role vlPFC plays in flexibility is to encode different visual features of stimuli and use that information to create representations of behavioural goals. However, the tasks employed in this chapter use identical stimuli, but different spatial locations. Even though the responses do not need to be visually discriminated based on features, vlPFC is important for successful behavioural planning. In favour of this is a study that showed that temporary inactivation of the vlPFC impaired reward-maximising behaviour by creating an attentional bias (Clarke et al., 2015). Animals needed to respond flexibly between two visually identical stimuli to maximise reward. One stimulus was accompanied by an aversive noise, the other was not. Inactivation of the vlPFC created a bias away from the more salient stimulus, which was accompanied by the aversive sound. This suggests that the attentional control vlPFC exerts over behaviour extends beyond visual features to include spatially guided sequences.

Temporary local inactivation of a brain area by infusions of $GABA_A$ and/or $GABA_B$-receptor agonists is a validated tool (Vaidya et al., 2019) that has been used to understand the contribution of specific brain areas in several species (e.g. vlPFC, marmosets, (Clarke et al., 2015), amygdala, rats (Muller et al., 1997) and cerebellum, macaques (Mason et al., 1998)). Muscimol and baclofen does however cause an effect across all cell types, it inactivates both inhibitory and excitatory cells. It is possible to more subtly affect neuronal signalling by using selective antagonists and agonists for receptors that are expressed more selectively across cell types. A candidate for such neuromodulation on the tasks presented here, could be the D_2-receptor, seeing as intra-vlPFC D_2 blockade has been shown to impair associative learning (Puig and Miller, 2015) and has also been implicated in mechanisms of attentional lability (Seamans and Robbins, 2010). Another candidate could be the 5-HT_{2A}-receptor which is widely expressed in the inferior frontal cortex (López-Giménez et al., 2001) and is involved in regulating pyramidal output of the PFC (Beique et al., 2007).

To conclude, data has been presented that extend previous lesion studies of the PFC by showing that temporary inactivation of the vlPFC impairs performance of spatial self-ordered sequences even in the absence of a delay component. Furthermore, it has been demonstrated that the greater the task flexibility, the stronger the impairment and that once a single sequence is heuristically learnt by repetition, vlPFC is no longer involved in performance of the sequence. Together these findings indicate a role for the vlPFC in in flexibly applying general behavioural principles to a larger set of problems.

4. Effects of serotonergic and dopaminergic neuromodulation of vlPFC on performance of variable response sequences

4.1. Introduction

In Chapter 3, data were presented that investigated the effects of inactivating vlPFC on performance of spatial response sequences. Inactivation of the vlPFC impaired performance of such sequences, when performance was required to be flexible. This inactivation of the vlPFC using $GABA_A$ and $GABA_B$-r agonists does however cause a non-specific effect across all cell-types containing GABA-rs. Very little is known about the chemical neuromodulation of vlPFC by the ascending monoamine systems, and even less in performance of spatial response sequences.

To the extent of the present author's knowledge, the only studies that have been published on prefrontal neuromodulation of spatial response sequences are selective serotonin and catecholamine depletion studies. Extensive prefrontal depletion of dopamine and noradrenaline by 6-hydroxydopamine (6-OHDA) in the marmoset showed no effect on a spatial self-ordered sequencing task (Collins et al., 1998). Prefrontal depletion of serotonin in the marmoset using 5,7-dihydroxytryptamine (5,7-DHT) was also without effect on spatial self-ordering performance although it did affect visual reversal learning (Walker et al., 2009a). Although depletion of serotonin or dopamine did not affect task performance, it cannot be ruled out that these systems play no role in the vlPFC mediation of executive functioning. Functional recovery is possible after monoamine depletion (Annett et al., 1992) and compensatory mechanisms can help rescue behaviour, especially since depletions were not total (for lPFC; 83.3% following 6-OHDA and 75% following 5,7-DHT). It is possible that more specific manipulations, such as acute microinfusions of selective dopamine or serotonin agents might be more effective at elucidating a potential role of these neurotransmitters in performance.

The few studies, mainly in the rhesus macaque monkey, that have investigated neuromodulation of the vlPFC, have focused on those portions of area 46 that are ventral of the arcuate sulcus, rather than area 47. One such study showed that the role vlPFC plays in associative learning and cognitive flexibility is dependent on both dopamine D1 and D2 receptors (Puig and Miller, 2015, 2012), those authors suggesting that D_2-r are more implicated in cognitive flexibility, while D_1-r are more implicated in associative learning.

As presented in the introduction, the D_2-r is expressed in the lPFC (de Almeida et al., 2008; Lidow et al., 1998) and based on a suggested role in regulation of cognitive flexibility (Floresco et al., 2006; Puig and Miller, 2015), would be a good target for intra-cerebral infusions into the vlPFC. Further to that, a study in rhesus demonstrated that systemic treatment with a D_2-antagonist, as-opposed to a D_1-antagonist, impaired performance of spatial self-ordered response sequences (Von Huben et al., 2006). Similar results have been observed in humans where high doses of the D_2-r antagonist sulpiride impaired performance on the most difficult blocks of trials in the CANTAB SWM task (Naef et al., 2017).

Another interesting candidate would be the $5HT_{2A}$-r. The $5HT_{2A}$-r is expressed in the vlPFC of primates (López-Giménez et al., 2001) and as described in Chapter 1, regulates pyramidal cell firing (Beique et al., 2007; Jakab and Goldman-Rakic, 1998). There is also evidence for the involvement of $5HT_{2A}$ -rs in performance of spatial response sequences. The hallucinogen LSD, which acts as a $5HT_{2A}$-r agonist, among other actions, impairs performance of the CANTAB SWM task and performance can be rescued with co-administration of the $5HT_{2A}$-r antagonist ketanserin (Pokorny et al., 2019).

The aim of the study presented in this chapter was to investigate the effect of local cerebral infusion of the D_2/D_3-r antagonist (S)-(-)-sulpiride (sulpiride) and the $5HT_{2A}$-r antagonist MDL-100,907 (m100907) on performance of the variable spatial self-ordered sequencing task which is dependent on the vlPFC. Any insights into the neuromodulation of vlPFC would further our understanding of the neural mechanisms behind prefrontal control of flexible behaviour and could ultimately lead to the development of novel pharmacological treatments.

4.2. Methods

4.2.1. Subjects

Four marmosets were the subjects of this chapter; two male and two female. For more information please see General Methods, **Table 2.1**.

4.2.2. Variable spatial self-ordered sequencing task

The task used to investigate the effect of $5HT_{2A}$ and D_2-r blockade was the 4-block probe variable self-ordered sequencing task presented in more detail in Chapter 3. The 4-block probe version was chosen over the non-probe version primarily because of a suggested role for lPFC D_2 receptors in error correction (Arnsten et al., 2015). The role of vlPFC D_2 and $5HT_{2A}$ in sequencing performance is however unknown and due to the exploratory nature of these infusions, the probe task was also chosen because a stronger impairment was seen following inactivation. Briefly, In the probe version of 4-block variable sequencing tasks animals perform, in a self-ordered fashion, spatial response sequences with 2 or 3 identical stimuli divided into 4 different blocks. Once a response to a stimulus was made it disappeared for a set amount of time, vanishing time. Animals could continue responding during that time. The first block of the task consisted of 2 circle trials with a vt of 0.5s. The remaining three blocks all contained 3 circle trials. Blocks 2 and 4 were identical with vts of 1s. Block 3 had a vt of 0.5s and thus was the most difficult block. The stimuli were randomly positioned before each trial based on an 8-position grid. On sessions in-between infusions, animals were punished for an erroneous response by trial abortion. On infusion sessions, errors were not punished and reward was given if all three responses were made. A trial was counted as not completed if on a given trial no response was made for 60 seconds.

4.2.3. Drug preparation and treatment

Sulpiride was prepared in two different concentrations, 3.75 µg/µl and 2.5 µg/µl. S-sulpiride was dissolved in 4000 µl of 0.1M HCl in saline. Solution was neutralised by slow addition of 1M NaOH until pH reached 7. Stock solution was diluted with phosphate-buffered saline (pbs) until a concentration of 10 µg/µl was achieved (a target volume of 10000 µl). Stock solution was filtered, aliquoted and stored at -20 for a maximum of 2 weeks. Stock solution aliquot

was thawed, diluted with pbs and filtered to desired concentration (3.75 or 2.5 µg/µl) before infusion. Vehicle was treated in the same way but contained no drug.

M100907 was prepared in four different concentrations (0.5, 1, 1.5 and 2 µg/µl). m100907 was made fresh before each infusion. Desired amount of drug was dissolved in 40 µl 0.1M HCl and dissolved to a goal volume of around 1000µl using pbs. Vehicle was 40µl 0.1ml HCl dissolved in 960 µl pbs.

Sulpiride and corresponding vehicle was infused at a rate of 0.5 µl/min over 1 minute. A 10-minute pre-treatment was allowed before animal was tested. All animals had vehicle and both concentration of drug infused.

M100907 and corresponding vehicle was infused at a rate of 0.5 µl/min for 1 or 2 minutes, depending on dose. A pre-treatment time of 12 minutes was allowed after infusion, prior to testing. A range of doses of M100907 was used between animals until they reached the maximum dose (2 µg) or a dose which caused them to disengage from testing. Disengaging was classified as having performed fewer than 50% of trials in block 4. All animals had 0.5 µl infusions prior to 1 µl infusions. The reason for the increase in volume rather than concentration is because of the solubility of the drug. **Table 4.1** shows the range of doses each subject in the study received.

M100907 doses and infusions						
Dose (µg)	Concentration	Volume	Subject 1	Subject 2	Subject 3	Subject 5
0	Vehicle	0.5 µl	√	√	√	√
0.25	0.5 µg/µl	0.5 µl	√		√	
0.5	1 µg/µl	0.5 µl	×	√		√
1	2 µg/µl	0.5 µl		√	√	√
0	Vehicle	1 µl		√	√	√
1	1 µg/µl	1 µl		√	√	√
1.5	1.5 µg/µl	1 µl		√		√
2	2 µg/µl	1 µl		×	√	×

Table 4.1 M100907 doses. All doses used for the M100907 infusion. The column Dose indicates the mass of drug delivered via infusion into the vlPFC. Calculated by the concentration and volume infused. One dose (1 µg) was given twice but with different concentration and volume. A green checkmark indicates that the subject was administered the dose while a blank cell indicates dose was not administered. A red x indicates that the dose was too high, animal disengaged from responding towards the end of the task.

Order of treatments were counterbalanced. Subjects 1 and 2 had infusions of sulpiride before M100907 and subjects 3 and 5 had infusions of M100907 before infusions of sulpiride. All animals had infusions of musbac into the vlPFC (see chapter 3) prior to the infusions presented in this chapter.

4.2.4. Data analysis

The experiment used a within-subject design. Testing data were collected in a Microsoft access database. Data were exported into Microsoft Excel (Office 365) and R studio (Version 1.2.1335, RStudio: Integrated Development for R. RStudio, Inc., Boston, MA). Statistical analysis was run in RStudio. Parts of the dataset were transferred from RStudio to create graphs in GraphPad Prism (Version 7.03 for Windows, GraphPad Software, La Jolla, California, USA).

Data were analysed using multiple linear mixed effects models with the r-package 'lme4' (Bates et al., 2015; Boisgontier and Cheval, 2016). Dose and block were fixed effects and subject was random effect. ANOVA was performed on the model to acquire p-values, using r-package 'lmerTest' (Kuznetsova et al., 2017). All doses for each animal were included in the analysis. For M100907 both replicate doses of 1 µg and both vehicle infusions, were included in the model. Data were presented graphically for each subject individually. To enable easier reading of graphical representations of the M100907 data, the doses that were replicate (1 µg of M100907 and the two vehicle infusions) were presented as mean values.

4.2.5. Surgical procedures

Following pre-operative training, cannulas were implanted to allow for central infusion of drugs. For more details on surgery, please see Chapter 2. Subject 1 had the cannulas implanted at an angle of 8°, subject 2 and 3 had the cannulas implanted at an angle of 10°, subject 5 had cannulas implanted at an angle of 9°. The reason for the discrepancy is that the angle was adjusted after histological analysis of animals in other studies.

4.3. Results

4.3.1. Histology

Histological analysis of cannula placements revealed that subjects 1, 3 and 5 had cannulae successfully implanted targeting vlPFC 47/12, see **Fig. 4.1**. Subject 2 is still pending histological analysis.

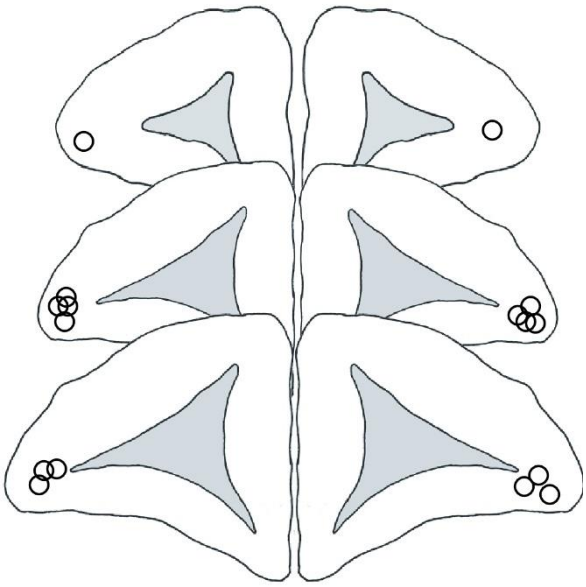


Figure 4.1 Cannula placements. Image illustrate end of cannula guides where complete or preliminary histological analysis have been performed Image adapted from Clarke et al (2015).

4.3.2. Blockade of vlPFC $5HT_{2A}$ receptors by infusion of M100907

Blockade of $5HT_{2A}$-r by infusion of M100907 into vlPFC impaired performance of self-ordered spatial response sequences, as reflected by a significant reduction in accuracy and increased numbers of errors per trial. The number of errors per erroneous trials were however not significantly different. Separate analysis was performed on individual error types, which revealed a strong trend for the number of continuous perseverative errors to increase. **Fig. 4.2** shows the effect of drug on accuracy for individual animals, while **Fig. 4.3** shows the effect

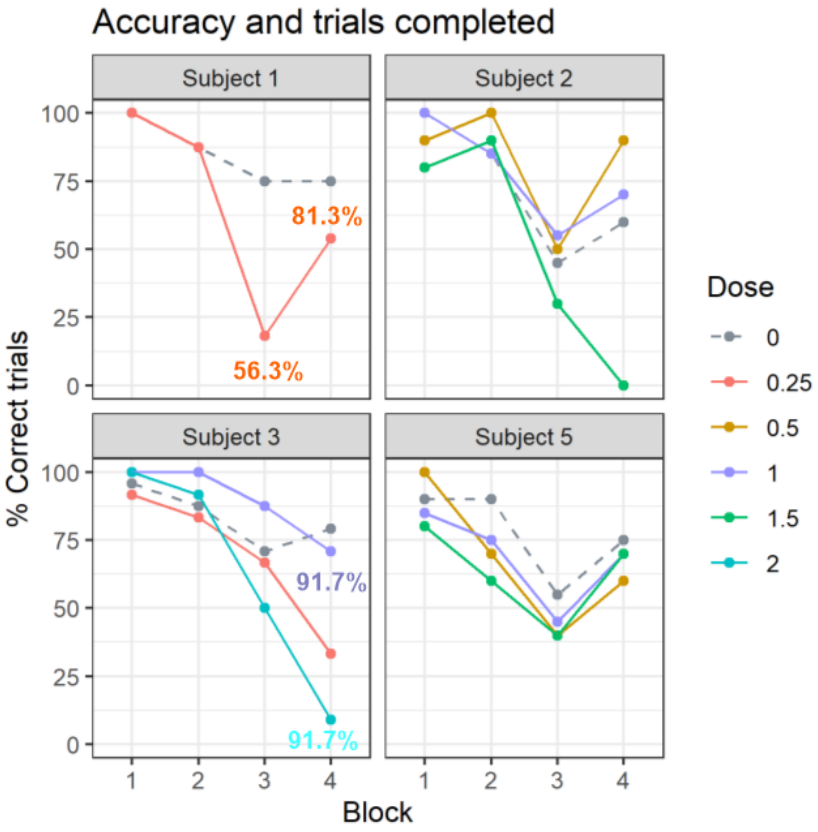


Figure 4.2 Accuracy and trials completed on the self-ordered spatial sequencing task following vlPFC 5HT$_{2A}$-r blockade. All graphs have the same colour coding for doses (µg). Replicate doses of vehicle (dose 0) and dose 1 are presented as a mean were applicable. Points show the percentage of completed trials where a sequence of three was performed without any error. If an animal omitted (by not making a response for 60 s) the trial was counted as not completed. If any trials where omitted in a block, the percentage of trials completed for that block is presented next to the accuracy value.

of drug on errors per trial as well as a breakdown into component errors for individual animals.

The highest doses of M100907 for subject 1, 2 and 5 were excluded from the analysis due to animals disengaging from testing altogether. At their individually highest dose, subjects 1, 2 and 5 all performed ≤ 30% of the trials in the fourth block.

Analysis of accuracy using a linear mixed effect model revealed a main effect of drug on accuracy ($F(5,52.572) = 3.583$, $p= 0.007$), where M100907 decreased accuracy. Qualitatively, this effect was strongest in the third and fourth blocks of the task, where accuracy was drastically decreased. There was also a main effect of block on accuracy ($F(3,53.006) =25.132$, $p < 0.001$), which just reflects that all animals showed lower accuracy on the more difficult

blocks as expected. On trials completed, there was a significant effect of drug ($F(5,53.235) = 3.793$, $p = 0.005$) and block ($F(3,51.403) = 5.728$, $p = 0.001$) as well as an interaction between the two ($F(15,51.403) = 3.491$, $p < 0.001$). Only two animals made omissions at the doses included in the analysis. On errors per trial there was also a significant effect of drug ($F(5,49.083) = 2.4487$, $p = 0.046$) and a significant effect of block ($F(3,53.103) = 8.6147$, $p < 0.001$). In 3 out of 4 subjects there was an increase in errors per trial in the third block of the task. On the fourth block this effect was true for all animals. On number of errors per

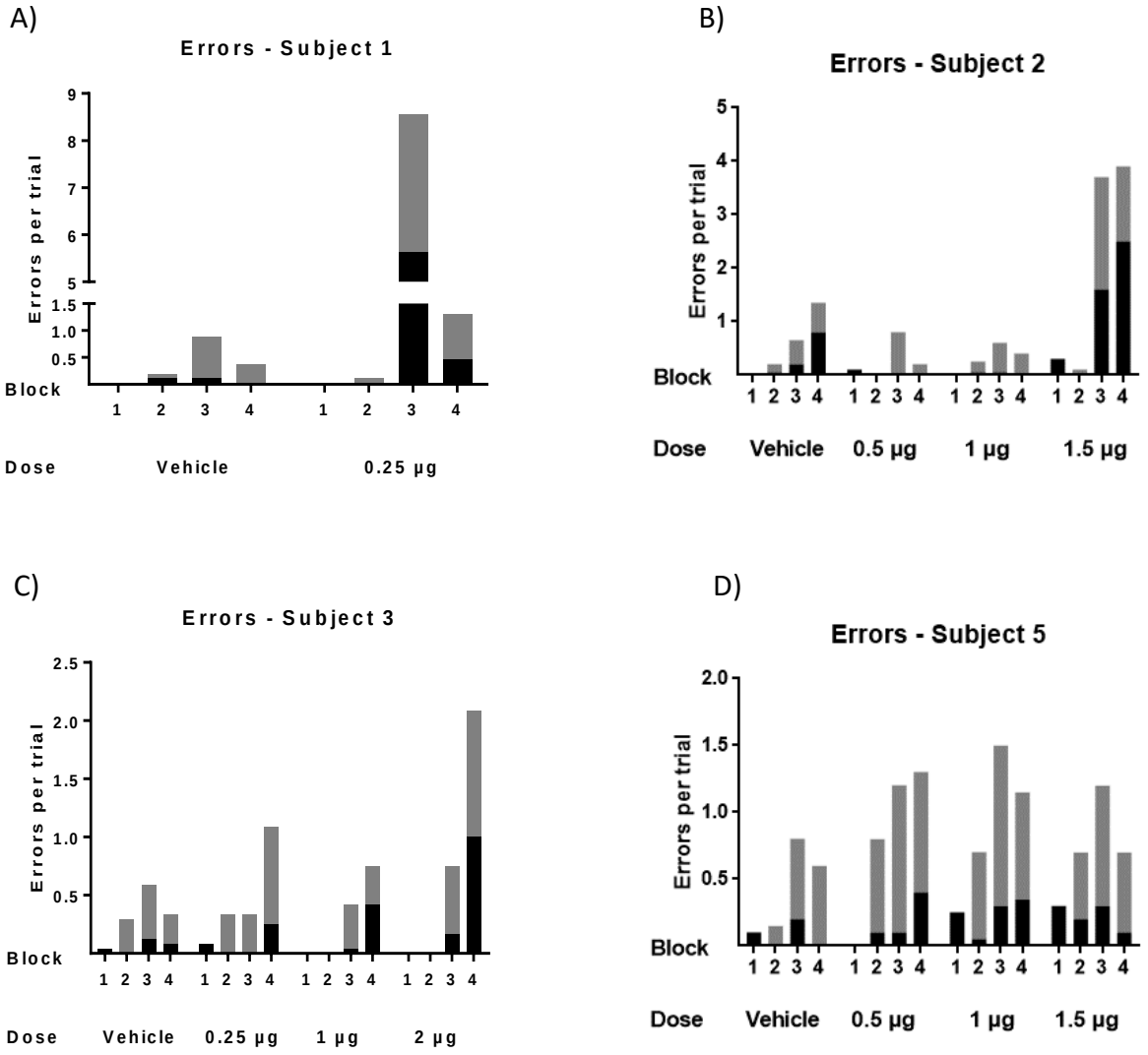


Figure 4.3 Errors per trial on the self-ordered spatial sequencing task following vlPFC $5HT_{2A}$-r blockade. Graphs illustrate the total errors per trial as well as the two different possible error-types performed by individual animals (A, B, C, D) after infusion of m100907 into vlPFC. The entire height of a bar represents the total numbers of errors per trial for each subject. Each bar is divided into recurrent (grey) and continuous (black) perseverative errors per trial, per block and dose. Replicate doses of vehicle (dose 0) and dose 1 are presented as a mean where applicable.

erroneous trials, **Fig. 4.4,** there was no significant effect of drug (F(5,53.769) = 1.289, p = 0.282) but there was an effect of block (F(3,53.337)= 7.776, p < 0.001). There was an effect on this measure on some doses and blocks, but it was less consistent across doses and animals. Examining individual error-types separately, there was a strong trend towards M100907 increasing the number of continuous errors per trial (F(5,44.714) = 2.359, p = 0.055). This effect was strongest in the fourth block of the task, but also present in the third block in most animals. There was a near significant effect of treatment on number of continuous perseverative errors per trial (F(5,43.690)= 2.348, p= 0.056). There was a significant effect of block on continuous errors per trial (F(3,52.063) = 3.674, p = 0.017) and a close to significant interaction between the two (F(15,52.063) = 1.807, p = 0.058). For the other possible error, recurrent perseverative errors, there was a trend towards drug to increase the number of recurrent errors per trial (F(5,40.320)= 2.295, p= 0.063) and a significant effect of block (F(2,39.312)= 9.660, p < 0.001).

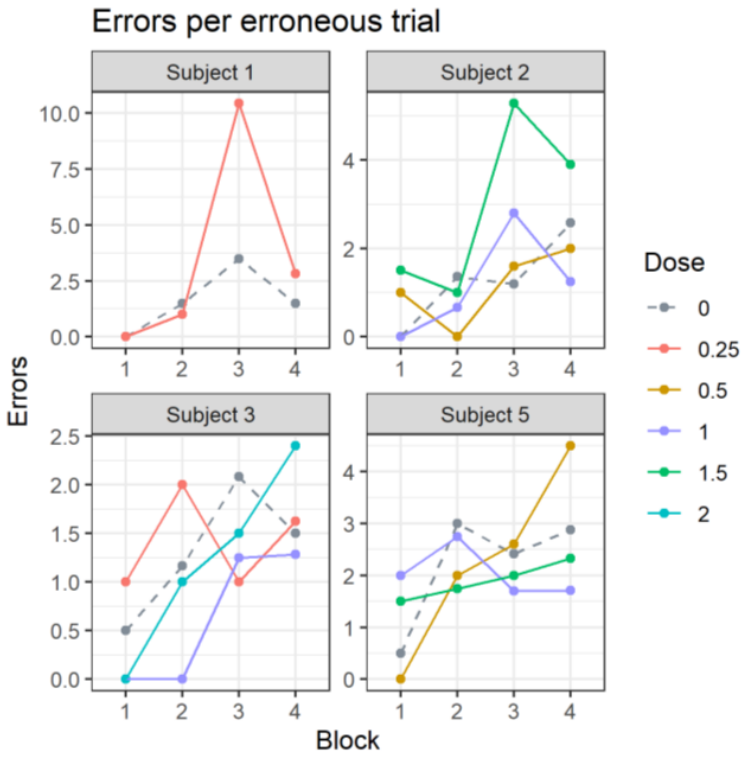


Figure 4.4 Errors per erroneous trials on the self-ordered spatial sequencing task following vlPFC 5HT$_{2A}$-r blockade. All graphs have the same colour coding for doses (µg). Replicate doses of vehicle (dose 0) and dose 1 are presented as a mean were applicable. Points with corresponding lines show the average number of errors made on trials which were completed with errors.

4.3.3. Blockade of vlPFC D_2 receptors by infusion of sulpiride

Blockade of D_2-r intra-vlPFC by infusion of sulpiride impaired performance of the spatial response sequencing task, as reflected by the significantly increased numbers of errors per trial and errors per erroneous trials. Accuracy was numerically affected for some animals, in some blocks, but only very nearly significantly impaired. **Fig. 4.5** shows the effect of infusion on accuracy and trials completed, while **Fig. 4.6** shows the effect on errors per trial and a breakdown into component errors and **Fig. 4.7** shows the effect on errors per erroneous trials.

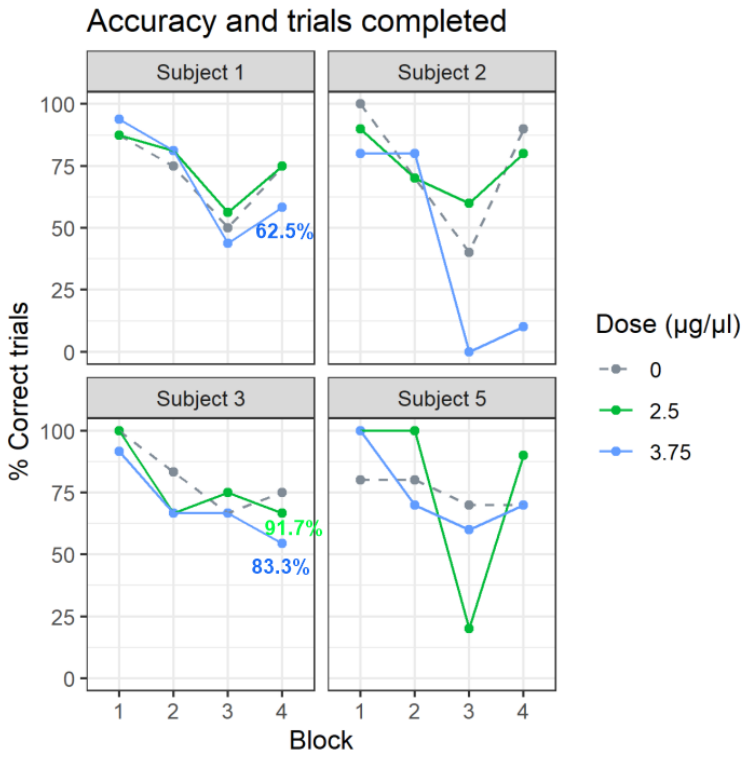


Figure 4.5 Accuracy and trials completed on the self-ordered spatial sequencing task following vlPFC D_2-r blockade. All graphs have the same colour coding for doses (µg). Points with corresponding lines show the percentage of completed trials where a sequence of three was performed without any error. If an animal omitted (by not making a response for 60 s) the trial was counted as not completed. If any trials where omitted in a block, the percentage of trials completed for that block is presented next to the accuracy value.

The linear mixed effects model showed a near significant effect of treatment with sulpiride on accuracy ($F(2,33)= 3.074$, $p= 0.059$) but a strong effect of block ($F(3,33)= 15.18$, $p < 0.001$). There was no interaction of block and treatment ($F(6,33)= 0.914$, $p= 0.496$). An accuracy decrease was very clear in two subjects (subject 5 and 2), but effects in the other two animals were less evident. On trials completed there was no significant effect of treatment ($F(2,33)= 1.918$, $p=0.162$) but there was an effect on block ($F(3,33)=2.936$, $p=0.04$). There was no interaction between the two ($F(6,33)= 1.91$, $p= 0.107$). Only two animals made omissions, all being in the fourth block and after treatment with sulpiride. On errors per trial there was a main effect of treatment ($F(2,33)=4.089$, $p= 0.025$) and block ($F(3,33)= 5.036$, $p= 0.006$), with no interaction between the two ($F(6,33)= 0.096$, $p= 0.462$). All animals showed an effect of

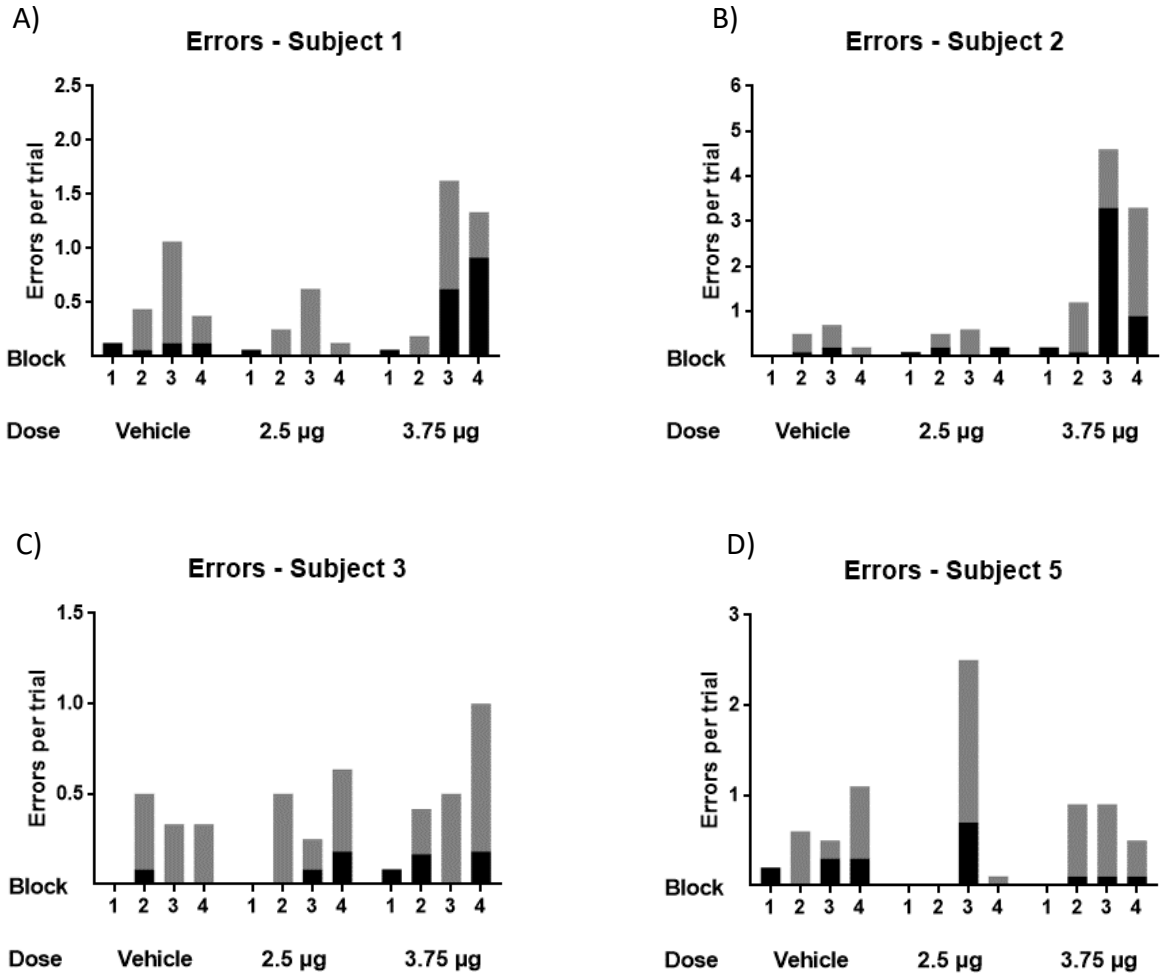


Figure 4.6 Errors per trial on self-ordered spatial sequencing task following vlPFC D2-r blockade. Graphs illustrate the total errors per trial as well as the two different possible error-types performed by animals after infusion of sulpiride into vlPFC (A, B, C,D). The entire height of a bar represents the total numbers of errors per trial for each subject. Each bar is divided into recurrent (grey) and continuous (black) perseverative errors per trial, per block and at each dose.

increased errors per trial in the third and/or fourth block. Sulpiride also significantly increased the numbers of errors per erroneous trials, ($F(2,33)= 4.192$, $p= 0.023$). There was also a main effect of block ($F(3,33)= 7.035$, $p < 0.001$) but no interaction between the two ($F(6,33)= 0.713$, $p= 0.641$). The effect of drug on errors per erroneous trials was consistent across animals in the third block of the task and in three out of four animals in the fourth block. Further analysis was performed looking at the two error types separately. For recurrent perseverative errors, there was a trend for treatment to increase recurrent perseverative errors ($F(2,27)= 2.994$, $p= 0.066$) but no significant effect of block ($F(2,27)= 1.282$, $p= 0.293$). On continuous perseverative errors, there was no significant effect of treatment ($F(2,33)= 2.546$, $p= 0.093$) or block ($F(3,33)=1.765$, $p=0.173$).

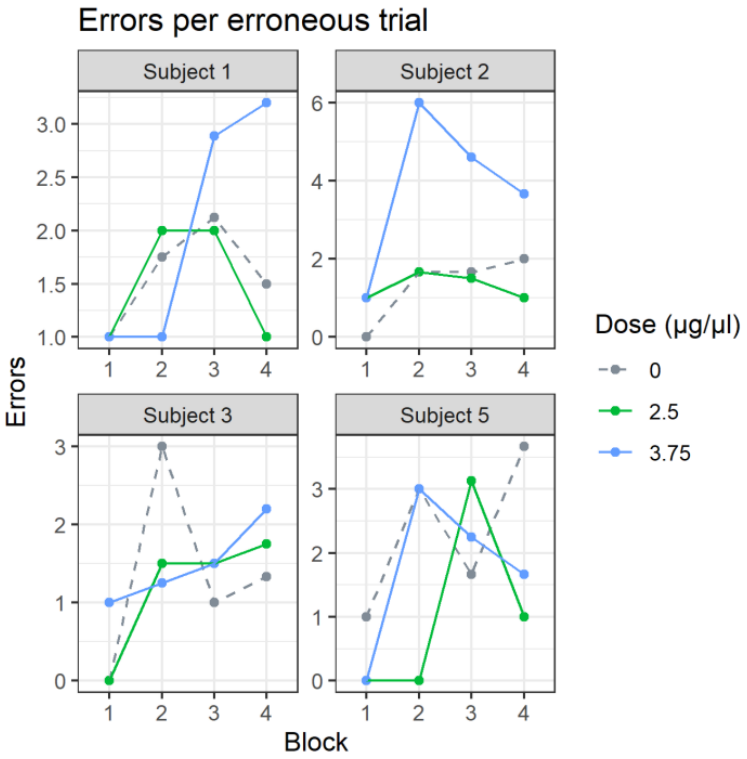


Figure 4.7 Errors per erroneous trials on the self-ordered spatial sequencing task following vlPFC D_2-r blockade. All graphs have the same colour coding for doses (µg). Points with corresponding lines show the average number of errors made on trials which were completed with errors.

4.3.4. Summary of results

Infusion of D_2-r antagonist sulpiride and $5HT_{2A}$-r antagonist M100907 both caused a behavioural impairment in performance of variable response sequences. Both drugs impaired the task by increasing the number of errors made. M100907 also significantly impaired task

accuracy, while there only was a trend for sulpiride to decrease accuracy (p=0.059). Both drugs increased the number of errors but did so in a slightly different manner. This is reflected in the number of errors made on erroneous trials. Infusion of M100907 left the number of errors performed on erroneous trials intact (p=0.282), while errors per erroneous trials were significantly increased following infusions of sulpiride. Separate analysis of the two error types showed no significant effects, but a trend for M100907 to increase the number of continuous perseverative errors and sulpiride a trend to increase the number of recurrent perseverative errors was evident.

4.4. Discussion

The overall behavioural impairments caused by M100907 and sulpiride were not clearly dissociable, but data suggest that they may have affected different behavioural mechanisms. M100907 produced a decrease in accuracy across subjects in which more errors were produced by an increase in the number of incorrect trials, but not necessarily by more errors being made on incorrect trials. For sulpiride the pattern of effects was almost opposite, with increased errors per incorrect trials across subjects, but less evidence of increases in the number of incorrect trials. This would suggest that these receptors may play a different role in regulation of behaviour. This could hypothetically be related to different roles in regulating pyramidal cell output. The lack of a clear behavioural dissociation could arise from receptor effects being exerted on partially overlapping circuitry.

These effects of the two chemical neuromodulatory treatments can be compared with the impairments observed following inactivation of vlPFC by infusion of $GABA_A$- and $GABA_B$-agonists (Chapter 3). For the latter, inactivation impaired task performance by decreasing accuracy, increasing the number of errors per trial, the number of errors per erroneous trials as well as the number of recurrent perseverative errors made, but leaving continuous errors per trials intact. All measures that were significantly impaired following inactivation are significant after treatment with sulpiride and/or M100907, except for recurrent perseverative errors, which was only near significant following administration of sulpiride (p=0.066). One difference between the more selective manipulations and inactivation was the tendency for M100907 to cause omissions towards the end of the task.

The most effective dose of M100907 had effects that were similar in 3 out of 4 animals. Subject 1 showed a strong sensitivity to lower doses than subjects 2, 3 and 5. All animals did however show a very similar response to the drug, with the highest individual dose causing a strong impairment that led to animal ceasing responding. Subject 2 never reached this threshold but did not complete all trials in the last block on the highest dose. A factor which complicates the interpretation of this data set is that different volumes had to be used. The highest concentration that was possible to achieve without making the solution overly acidic was 2 µg/ul. This led to difficulties, because a higher dose in the same volume would have been unachievable without altering the formulation. The doses used in this study were similar to or slightly higher than what has been used in rodents (Boulougouris and Robbins, 2010; Pockros et al., 2011; Winstanley et al., 2003).

All animals had the same two doses of sulpiride and even though there was a significant impairment on errors per trial and errors per erroneous trials, there was only a close to significant effect of decreased accuracy. It is possible that animals were differentially sensitive to sulpiride and that a higher dose would have caused an impairment in the animals which did not show a decline in accuracy. A stronger dose (0.5 µl of 10 µg/ul sulpiride) was tested in subject 1 but caused adverse effects, ruling out the use of higher doses for any other subject. However, the doses used were in line with those used in the rodent PFC (Druzin et al., 2000; Granon et al., 2000; Seamans et al., 1998), but lower than doses used in the rhesus dlPFC (Sawaguchi and Goldman-Rakic, 1994). Even though sulpiride shows the greatest affinity for the D_2-r, it also acts as an antagonist at the D_3-r. Affinity for the D_2-r is two times greater than the affinity for D_3-r (Caley and Weber, 1995). The effect is likely to be attributed to an effect on D_2-rs, because D_3-r expression in the PFC is scarce (Lévesque et al., 1992). However, it cannot be ruled out completely without single- or co-administration of a D_3-antagonist.

The impairment following $5HT_{2A}$-r blockade appeared in the third block of the task and was present in the fourth block also. According to some measures, the impairment was even more severe in the last block of the task. The fourth block was identical to the second block of the task, so this strong impairment in block 4 needs careful interpretation. It could be argued that the late impairment is a delayed effect of drug. However, the waiting time used here was longer than the waiting time in other studies, which tested immediately following infusion (Boulougouris and Robbins, 2010; Pockros et al., 2011; Winstanley et al., 2003). Previous

unpublished data in the marmoset from this laboratory support an almost immediate effect of M100907 intra-PFC, where physiological cardiac rhythm changes were observed immediately following infusion. Another explanation could be that the impairment in the fourth block was caused by a demotivation from poor performance in block 3. Previous findings do however not support the notion that it is attributed to decreased overall motivation as inactivating vlPFC by infusion of musbac did not reduce the number of responses marmosets made for reward (Clarke et al., 2015). It could be an effect specific to blockade of vlPFC 5-HT_{2A} as opposed to inactivation, but it is more likely the impairment is related to attention. As described in the introduction, pyramidal $5HT_{2A}$ receptors have been suggested to facilitate sensory inputs. The task requires animals to adapt to alternating difficulties between blocks and the impairment could conceivably be in shifting between these task sets. Once animals reach the third block, the stimuli reappear faster after selection, which increases the attentional requirements of the task. They were unable to adapt to the more difficult trials and once impaired unable to recover behaviourally at the last block. This theory could be tested in future experiments, by using the 1-block variable sequencing task (presented in Chapter 3), in which animals only experience one level of task difficulty. If the animals were not impaired, the impairment would simply have been in shifting behaviour in environments with alternating difficulties. If impairment persists, it is more likely to reflect the inability to allocate appropriate attentional demand on the difficult trials. An impairment in attentional processes is supported by previous literature showing a role for vlPFC in shifting (Dias et al., 1996) and attentional selection (Clarke et al., 2015). Contradictory to this hypothesis is the successful transition between two circle and three circle trials (between block 1 and 2). However, at this stage of testing animals were very experienced with the task and performance of 2-circle trials may no longer have required vlPFC activity.

The mechanism behind the impairment following D_2-r blockade is more likely to have another explanation. Sulpiride did not significantly decrease accuracy, even though there was a clear decrease in accuracy for two animals. The number of errors animals made were however significantly increased, driven by an increase in the errors made on erroneous trials. This indicates that the impairment is in the ability to correct responding once errors have been performed. The dual-state theory on prefrontal dopamine suggests that the prefrontal $D_{1\text{-}r}$ is important for maintaining representations while the D_2-r, act to lower the energy barrier

between states, and enables flexible and fast switching between representations (Durstewitz and Seamans, 2008). According to the dual-state theory, infusions of sulpiride would cause a shift to a D_1-state, which would make subjects unable to flexibly adapt their behaviour. The theory fits with the observed data, animals were impaired at flexibly processing feedback of superfluous erroneous responses to stop excessive responding on trials where the representation (potentially of a behaviour plan) were incorrect. The sulpiride data presented in this chapter are generally in agreement with a study in the rhesus macaque demonstrating an impairment in cognitive flexibility following infusion of raclopride into the vlPFC (Puig and Miller, 2015). Systemic treatment with D_2-antagonist impaired performance of spatial response sequencing in both primates (Von Huben et al., 2006) and humans (Naef et al., 2017). Comparison across tasks are however quite difficult, due to the relatively small detail of behavioural data presented. Von Huben et al present no data on errors but show that accuracy declined, particularly on the more difficult 4 stimuli trials. This matches up well with the findings in Naef et al, which show that human participants show impaired performance on the more difficult problems. They also show that sulpiride increased the number of between search errors on these trials, while leaving a strategy measure intact. This could indicate a similarity between their findings and the findings in this chapter and suggests that vlPFC is one neural locus (of possibly several) for this effect.

As presented in Chapter 1, The psychiatric disorder schizophrenia is treated with antipsychotics, which act on D_2-r and 5-HT_{2A}-r. Antipsychotics generally treat positive symptoms (hallucinations, delusions) well but the effect on cognitive symptoms are less consistent (Tandon, 2011). The restoration of executive functioning is important for recovery (Green et al., 2000) and impaired performance of action sequences would negatively affect daily living activities in schizophrenia (Semkovska et al., 2004). This chapter suggests that antipsychotics could impair action sequencing by its action on 5-HT_{2A} and D_2-rs, which in turn might worsen community outcome.

Blockade of prefrontal 5-HT_{2A}-r suppresses cortical pyramidal cell output (Beique et al., 2007) and thus could the impairment be theorised to be a lack of input from vlPFC to connecting areas. To fully understand the performance of spatial self-ordered sequences, the neural network involved needs to be identified. An area of interest to study would be the caudate

nucleus, an area with dense connections with vlPFC (Roberts et al., 2007) that is known to be involved in the performance of spatial response sequences (Miyachi et al., 1997).

To conclude, data have been presented which demonstrate that the vlPFC control over performance of spatial self-ordered sequences is dependent on both dopaminergic and serotoninergic neurotransmission. The impairments caused by blockade of D_2 and 5-HT_{2A}-rs were dissociable. Blockade of 5-HT_{2A}-r impaired accuracy and increased errors generally, while blockade of D_2-r played a stronger role in preventing the correction of behaviour once erroneous responses were made. The roles of two receptors were however not completely dissociable and so highlight the complex modulation of executive functioning in the PFC.

5. Effects of blocking input to the caudate on performance of variable and fixed self-ordered response sequences

5.1. Introduction

In the first two experimental chapters of this thesis, investigations were made into the involvement of vlPFC in performance of spatial self-ordered response sequences. However, little is known about the neural circuitry involved in performance of spatial self-ordered response sequences, especially in connection to the vlPFC. vlPFC has extensive connections with both cortical and subcortical areas in the marmoset (Roberts et al., 2007) so a potential target for investigation of the neural circuitry needs to be considered. A prime candidate for investigation would be the dorsal striatum, a part of the basal ganglia with dense connections from vlPFC (Roberts et al., 2007). The dorsal striatum is also known to be involved in performance of externally guided spatial response sequences (Miyachi et al., 1997). The dorsal striatum contains two areas of interest for the study, the caudate nucleus and the putamen. Studies in rodents on the areas homologous to caudate and putamen show that a dichotomy exists in their control of instrumental behaviour. Caudate is involved in goal-directed control of behaviour through learning of action-outcome associations (Yin et al., 2005) while putamen is involved in control of habitual behaviour (Yin et al., 2004). A similar relationship in control of behaviour can also be found in skill learning, where caudate is involved in early learning of a skill, while putamen plays a bigger role in performance of a learned skill (Yin et al., 2009). Yin et al also show that initial skill learning is dependent on D_1 and D_2 receptor activation while performance after extended training is only dependant on D_2 receptors.

Findings in rhesus macaque on performance of spatial response sequences generally agree with these findings, where separate inactivation of anterior putamen and anterior caudate impair early performance of a 2x5 task, previously described in the Introduction. In contrast,

only posterior putamen impairs late performance (Miyachi et al., 1997), indicating that caudate is only involved in performance before the sequence is learnt as a motor skill.

In contrast to the 2x5 task described above, the variable sequencing task used in this thesis was designed to maintain flexible performance over extended periods of time by alternating stimuli positions and being self-ordered. This argues for an involvement of the caudate in performance of the variable spatial self-ordered sequencing task, while the putamen is hypothetically more likely to be involved in performance of sequences that more easily can be reduced to motor skills, like the fixed self-ordered sequencing task.

Data were presented in chapter 3 that illustrate vlPFC dependence on performance of variable self-ordered sequences as opposed to fixed sequences. vlPFC-caudate signalling could be crucial for this flexible responding. This hypothesis could be tested using infusions of a variety of pharmacological agents into the caudate. One method would be inactivating the area with a GABA-agonist, but previous studies have shown that a range of doses is necessary because of individual variability in response to intra-striatal muscimol in marmosets (Jackson et al., 2019), which could cause practical difficulties. Another approach would be to block prefrontal cortical input. Prefrontal input to the striatum is glutamatergic (Fonnum et al., 1981) and could be partially blocked using infusions of the AMPA antagonist cyanquixaline (CNQX). This approach has proved to be successful in the dorsal striatum of rats (Furlong et al., 2014) and unpublished data from this laboratory indicate that infusion of CNQX is viable for investigating prefrontal-striatal relationships in a stable dose-response manner (Duan et al., manuscript in preparation). A third method could be to pharmacologically manipulate dopamine receptors in the caudate, which is known to have an effect on skill learning (Yin et al., 2009).

In this chapter data will be presented that investigated the effects of blocking glutamatergic input in caudate on performance of both variable and fixed spatial response sequences. See **Fig. 5.1** for an overview of tasks. Glutamatergic AMPA input was blocked by infusion of CNQX in an area of the caudate that primarily has cortical input from the vlPFC. There are however overlap from other cortical projections and likely also other subcortical projections. The hypothesis was that blocking input would impair performance of variable response sequences, but leave performance of fixed sequences intact, similar to inactivation of the

vlPFC. A further pilot study was also performed in one subject which investigated the effect of D_2-r blockade on performance of variable response sequences.

5.2. Methods

5.2.1. Subjects

Three marmosets, one male and two female, were the subjects of the experiments described in this chapter. For more details, please see General Methods chapter, **Table 2.1**.

5.2.2. Variable spatial self-ordered sequencing task

All subjects performed a variable spatial self-ordered sequencing task, identical to the 1-block variable sequencing task described in more detail in Chapter 3, 3.2.3.2. In this chapter the task is however only referred to as the variable spatial self-ordered sequencing task. Briefly, in the 1-block variable self-ordered sequencing task animals needed to perform 30 3-circle trials. Stimuli were identical and once a response was made, the selected stimulus disappeared for a vt of 0.5s. Animals performed the sequence in a self-ordered fashion and were given liquid reward if they performed a sequence of three without repeating any response. If a response was repeated the trial was aborted. Stimuli positions were randomised for each trial based on an 8-position grid (**Fig. 5.1 C**).

5.2.3. Fixed spatial self-ordered sequencing task

All subjects also performed the fixed spatial self-ordered sequencing task, previously described in Chapter 3, 3.2.3.3. Briefly the task was identical to the variable spatial self-ordered sequencing task described above with the exception that the sequence remained constant over days. A fixed sequence of 3 circle positions (unique for each animal) was performed over at least ten days before infusions.

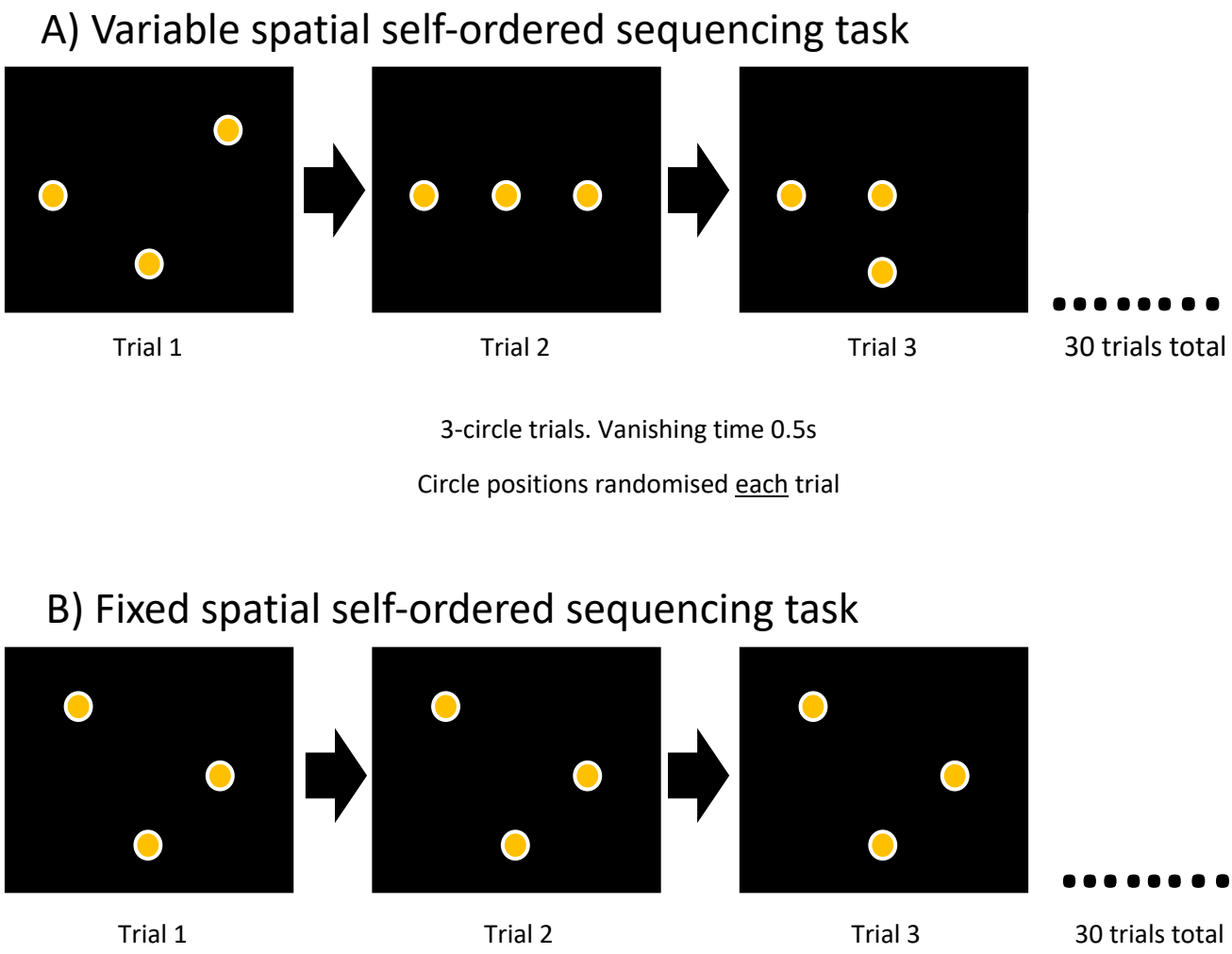


Figure 5.1 Task overview. Figure illustrates an overview of the tasks used in this chapter. The variable (A) and fixed (B) sequencing tasks. Stimuli location was randomised for each trial in the variable sequencing task based on an 8-position grid (C). For the fixed sequencing task one sequence generated from the 8-position grid was performed over days.

5.2.4. Data analysis

All studies used a within-subject design. Testing data were collected in a Microsoft access database. Data were exported into Microsoft Excel (Office 365) and R studio (Version 1.2.1335, RStudio: Integrated Development for R. RStudio, Inc., Boston, MA). Data were managed in RStudio and Excel before statistical analysis was run in GraphPad Prism (Version 7.03 for Windows, GraphPad Software, La Jolla, California, USA).

All data had one factor, drug treatment, with two levels and were analysed using a two-tailed paired t-test. Data were presented with mean comparison and standard error of the mean. $P < 0.05$ was used for statistical significance and denoted with an asterisk *.

5.2.5. Surgical procedures

All subjects had permanent indwelling cannulas implanted targeting both the vlPFC and the caudate bilaterally. For more details on surgery, see Chapter 2. Only caudate data are presented in this chapter, but animals also had vlPFC manipulations, presented in Chapter 3. Caudate cannulas were implanted at +11 ap, ± 3.3mm LM. Coordinates were chosen to allow for infusions into an area of the caudate which primarily had prefrontal inputs from the vlPFC (Roberts et al., 2007). **Fig. 5.2** illustrates the cannula target area.

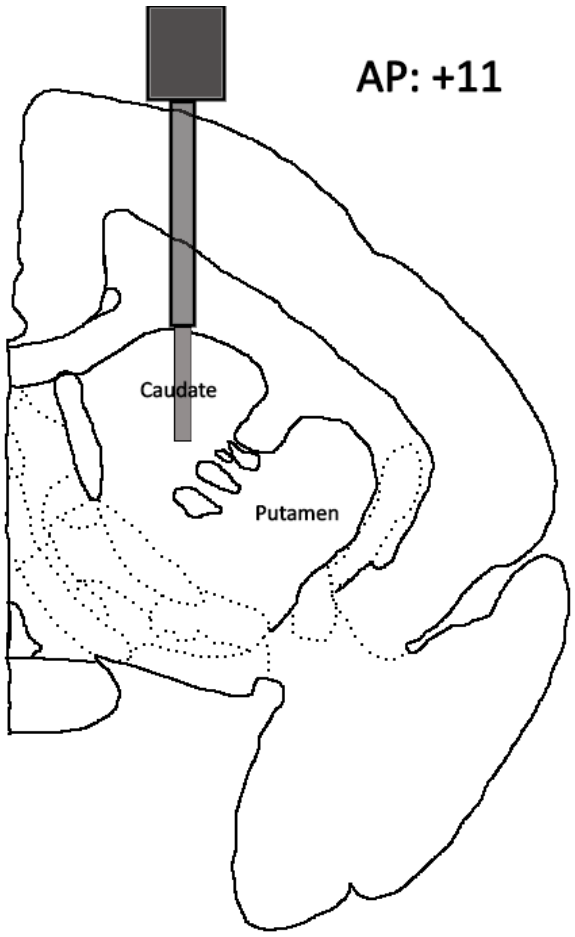


Figure 5.2 Caudate cannula placements. Image illustrates the placement of the caudate cannula. Cannula was mounted at AP +11, LM ±3.3. Grey boxes illustrate in descending order, cannula pedestal, cannula guide and injector for infusion. Boxes are not to scale, but end of injector indicates target infusion area. Image adapted from Paxinos et al (2012).

5.2.6. Drug preparation and treatment

All animals had infusions of the AMPA-receptor antagonist CNQX and the corresponding vehicle saline into caudate. Two different volumes were used of both drug and vehicle for each task, 0.3 µl over 1 minute and 1 µl over 2 minutes. CNQX was prepared by dissolving it in saline to a concentration of 1mM. 1mM was the final concentration used for all infusions. CNQX was filtered, aliquoted and stored at -20 for a maximum of 30 days. Aliquots were thawed immediately before testing. All injectors used extended +2 mm outside of the guide, except for subject 7, which needed a +2.5mm injector on the right guide, due to the right cannula being mounted higher.

Animals had infusions of 0.3 µl CNQX and vehicle on the variable sequencing task, before having 0.3 and 1 µl of CNQX and vehicle on the fixed sequencing task, before being retrained on the variable sequencing task and having infusions of 1 µl CNQX and vehicle.

Subject 6 also had infusions of (S)-(-)-Sulpiride (sulpiride) in doses of 10, 60 and 600 ng into the caudate. The drug was prepared before infusions by dissolving the required weight of sulpiride in 20 µl HCl before dilution with PBS to a goal volume of 1000 µl. The drug was filtered after preparation. Infusion was done at a rate of 0.5 µl/min for 2 minutes. A 10-minute waiting time was allowed after infusion before testing.

5.3. Results

5.3.1. Histology

Histological analysis based of cannula placements revealed that all guides targeted the caudate nucleus, see **Fig. 5.3**. Subjects 6 did however have their cannula implanted at around -0.5 mm from AP target. Based of connectivity from Roberts et al. (2007), infusions in all animals targeted an area with input from the vlPFC.

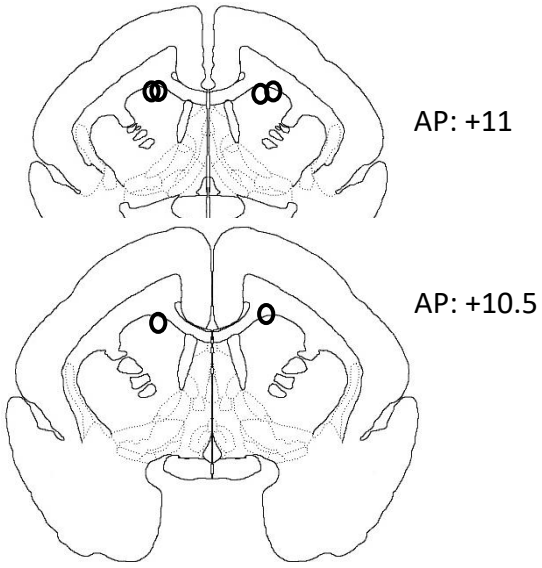


Figure 5.3 Cannula placements. Image illustrate approximate end of cannula guide for subjects at either AP+ 11 or AP+ 10.5. Image adapted from Paxinos et al (2012).

5.3.2. Variable sequencing task – 0.3 µl infusions

Fig. 5.4 shows that Infusions of 0.3 µl of CNQX into the caudate did not significantly alter any behavioural scores but did decrease continuous perseverative errors in 2 out of 3 animals.

Animals did not significantly differ in the number of correct trials made without errors, a two-tailed paired student's t-test showed no significant effect of CNQX infusion on accuracy (**Fig. 5.4 A**), mean ± SEM difference for CNQX – Saline = 5.55 ± 7.78, p= 0.549.

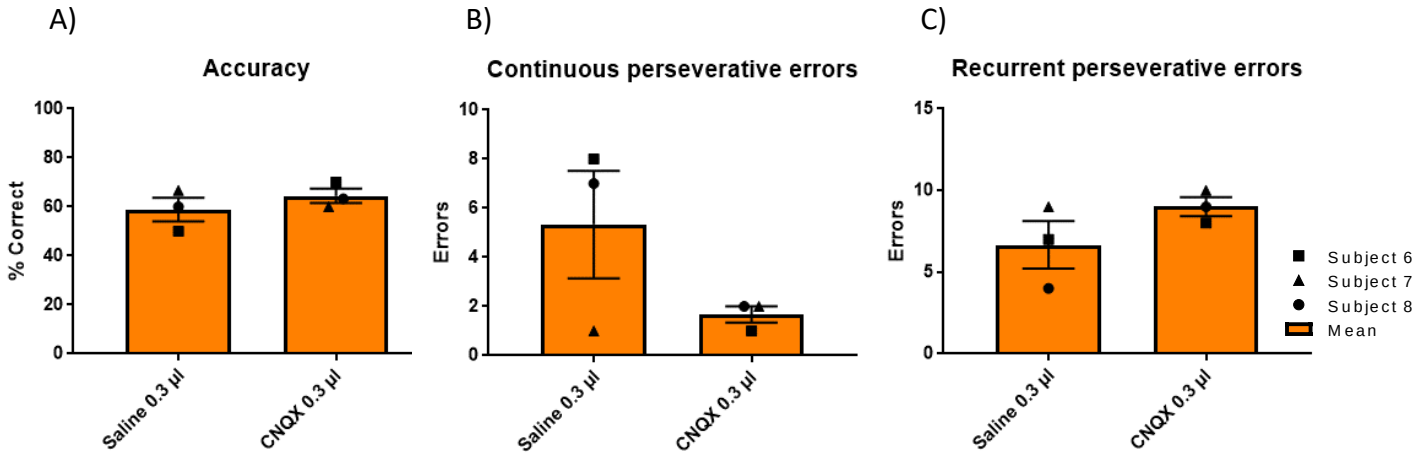


Figure 5.4 Performance of the variable sequencing task following infusion of 0.3µl 1mM CNQX into caudate. The two bars represent the mean for the different manipulations, with individual data-points. Error bars represent standard errors. All graphs have the same key, presented on the far right. A) There was no significant effect of treatment on accuracy. B) Two subjects showed a strong decrease in the number of continuous perseverative errors, while the last subject made almost no continuous perseverative errors under any manipulation. C) There was no statistically significant effect on recurrent perseverative errors, although all animals showed a numerical increase, it was small in two out of three animals.

Two different errors are possible on the tasks presented in this chapter, separate analysis was performed on both error-types to ensure that even though accuracy remained unchanged, there was no difference in the error profile between manipulations. Two animals (subject 6 & 8) showed a decrease in the number of continuous perseverative errors. However, the third animal (subject 7) performed very few continuous perseverative errors under both conditions. As a consequence, there was no significant effect on continuous perseverative errors (Mean ± SEM difference for CNQX – Saline = -3.667 ± 2.40, p= 0.267) (**Fig. 5.4 B**). To investigate this effect further, baseline continuous perseverative errors for Subject 6 and 8 were analysed for six sessions before CNQX infusion (with first testing day of the week

excluded). Six sessions were selected to capture the week with infusions, but also capture the later days of the previous week. These six sessions included the saline infusion for both animals. **Fig. 5.5** shows that for subject 6, the mean continuous perseverative errors ± 95% confidence interval was 5.5 ± 1.314 while for subject 8, the mean continuous perseverative errors ± 95% confidence interval was 5.16 ± 2.341. Both CNQX infusions days were below the 95% confidence interval during baseline. For the other error type, recurrent perseverative errors (**Fig. 5.4 C**), two subjects (6 and 7) showed a small increase, but one (subject 8) subject showed a strong increase in recurrent-perseverative errors. There were however no significant differences between infusions of CNQX and saline, mean ± SEM difference for CNQX – Saline = 2.33 ± 1.33, p= 0. 222.

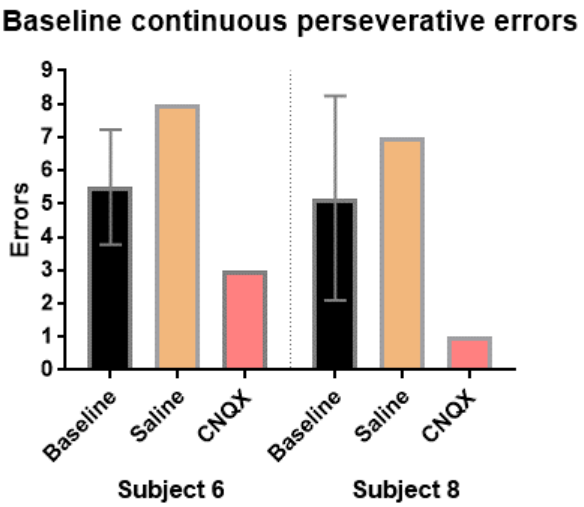


Figure 5.5 Baseline continuous perseverative errors compared to 0.3 µl infusions of 1mM CNQX and saline into caudate. Black graph represents the mean number of continuous errors for 6 testing sessions (including saline) prior to CNQX infusion, errors bars represent the 95% confidence interval. The yellow bar represents the absolute number of continuous errors on the testing session following saline infusion, while the red bar represents the CQNX testing session.

5.3.3. Fixed sequencing task – 0.3 and 1 µl infusions

Fig. 5.6 shows that treatment with 1mM CNQX in 0.3 µl did not significantly alter any behavioural scores as compared to the corresponding vehicle infusions. After having performed an initial 0.3 µl infusion with no effect on either the fixed or variable sequencing task, a decision was made to increase the volume to 1 µl, to target a larger area of the caudate.

Fig. 5.7 shows that there were no significant effects on behavioural scores following a 1 µl infusion of 1mM CNQX.

There was no clear impairment or improvement on accuracy for either infusion volume (**Fig. 5.6 A**). For 0.3 µl infusions, the mean ± SEM difference of accuracy, CNQX – Saline = -6.67 ± 3.33, p= 0.184. For 1 µl infusions, mean ± SEM difference on accuracy for CNQX – Saline = -7.77 ± 9.69, p= 0.51 (**Fig. 5.7 A**).

Separate error types were also investigated, for continuous perseverative errors at the lower volume, all animals showed increases in the number of errors, but two animals only had very small increases (**Fig. 5.6 B**). Mean ± SEM difference for 0.3 µl infusions, CNQX – Saline = 2 ±

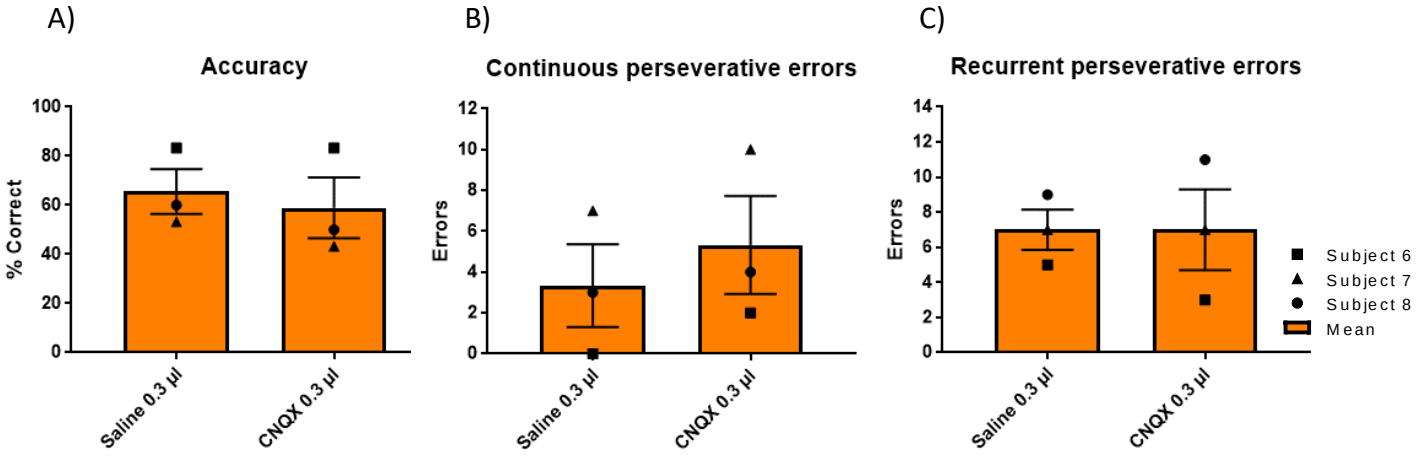


Figure 5.6 Performance of the fixed sequencing task after infusion of 0.3µl 1mM CNQX into caudate. The two bars represent the mean for the different manipulations, with individual data-points. Error bars represent standard errors. All graphs have the same key, presented on the far right. A) There was no significant effect of treatment on accuracy. B) Although all subjects increased the number of continuous errors the effect was small in two animals and there is no significant increase in continuous perseverative errors. C) There was no statistically significant effect on recurrent perseverative errors.

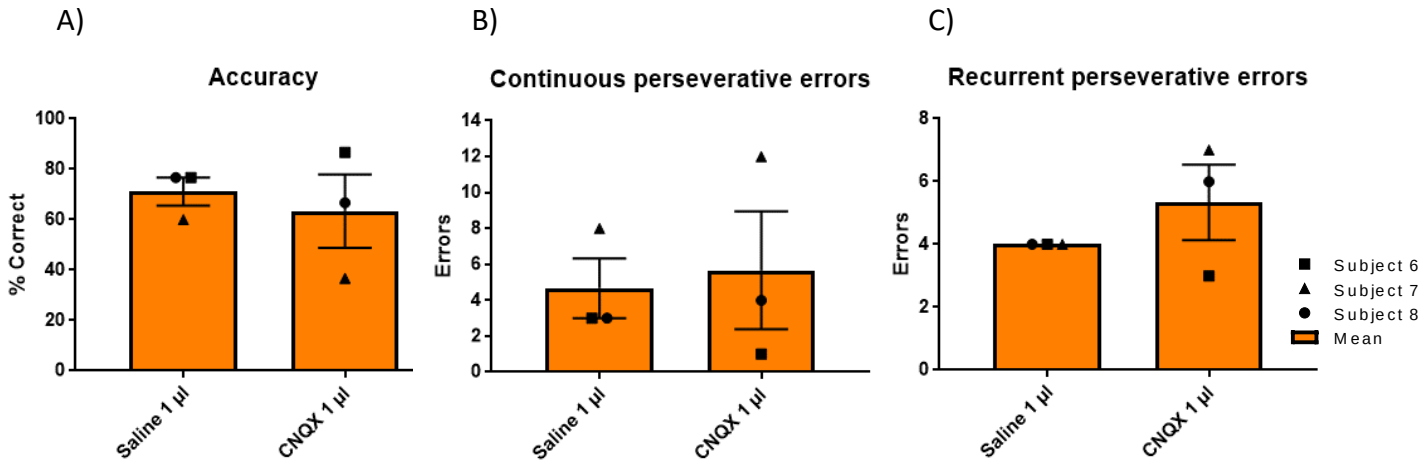


Figure 5.7 Performance of the fixed sequencing task after infusion of 1µl 1mM CNQX into caudate. The two bars represent the mean for the different manipulations, with individual data-points. Error bars represent standard errors. All graphs have the same key, presented on the far right. A) There was no significant effect of treatment on accuracy. B) There was no clear effect on continuous perseverative errors. C) There was not a statistically significant effect on recurrent perseverative errors.

0.58, p= 0.074. For 1 µl infusions there was also an increase of mean continuous perseverative errors, but the increase was driven by one animal, mean ± SEM difference for 1 µl infusions, CNQX – Saline = 1 ± 1.73, p= 0.622 (**Fig. 5.7 B**). For the other error type, recurrent perseverative errors, there was no clear effect of the lower volume of CNQX, mean ± SEM difference for 0.3 µl infusions, CNQX – Saline = 0 ± 1.16, p= 0.99 (**Fig. 5.6 C**). In agreement with the 0.3 µl infusion, there was no clear effect of 1 µl of CNQX infusions either (**Fig. 5.7 C**), two animals did however show an increase in recurrent perseverative errors, mean ± SEM difference for 1 µl infusions, CNQX – Saline = 1.33 ± 1.20, p= 0.382.

All animals heuristically adapted a behavioural strategy to solve the fixed sequencing task. Subjects 6 & 7 adopted a counter clockwise/clockwise strategy to solve the task, while subject 8 adopted a strategy where she almost exclusively started responding to a particular stimulus. Qualitative analysis of responding showed that the strategy uses of subject 6 & 8 remained very stable over sessions whereas subject 7 showed greater flexibility in his performance across sessions, changing from a clockwise to counter clockwise strategy. Subject 7 also showed less reliability in his performance of the task. The percentage a strategy was used on infusions days were compared for drug and vehicle. **Fig. 5.8** shows that manipulations had no significant effect on use of strategy for either infusion volume. Mean ± SEM difference of strategy use for 0.3 µl infusions (**Fig. 5.8 A**), CNQX – Saline = 12.04 ± 16.82, p= 0.545. Mean ± SEM difference for 1 µl infusions (**Fig. 5.8 B**), CNQX – Saline = 0.292 ± 2.03 p= 0.899. For a

more detailed view on performance and strategy use on the fixed sequencing task, please see **Supplementary figures 5.1, 5.2 and 5.3.**

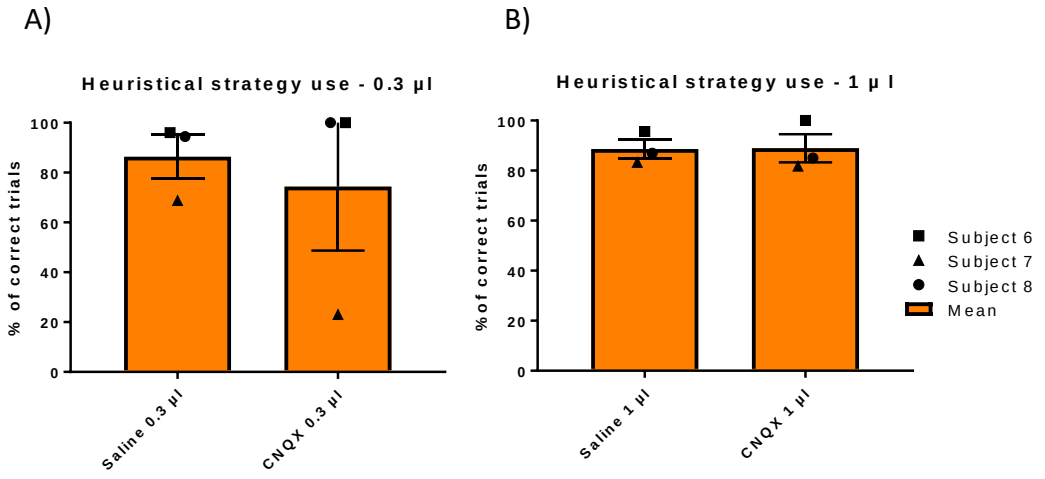


Figure 5.8 Strategy use on the fixed sequencing task after infusion of 0.3 µl and 1µl 1mM CNQX into caudate. Graphs present the use of strategy as a percentage of strategy used on correct trials. The two bars represent the mean for the different manipulations, with individual data-points. Error bars represent standard errors. All graphs have the same key, presented on the far right. A) There was no significant main effect of treatment on strategy use following 0.3 µl infusions B) There was no significant main effect of treatment on strategy use following 1 µl infusions.

5.3.4. Variable sequencing task – 1 µl infusions

After investigating 1 µl infusions on the fixed sequencing task, but not on the variable sequencing task, animals were re-trained on the variable sequencing task and infusions of CNQX and saline in 1µl were performed. **Fig. 5.9** shows that 1 µl infusions of CNQX did not significantly alter any behavioural score as compared to saline.

A two-tailed paired Student's t-test showed no significant effect of CNQX infusion on accuracy (**Fig. 5.9 A**), mean ± SEM difference for CNQX – Saline = 2.23 ± 7.29, p= 0.788.

Looking at individual error types separately, there was no clear effect on continuous perseverative errors (**Fig. 5.9 B**), mean ± SEM difference, CNQX – Saline = -0.33 ± 1.45, p= 0.839. There was also no clear effect on recurrent perseverative errors (**Fig. 5.9 C**), mean ± SEM difference, CNQX – Saline = -0.33 ± 0.88, p= 0.742.

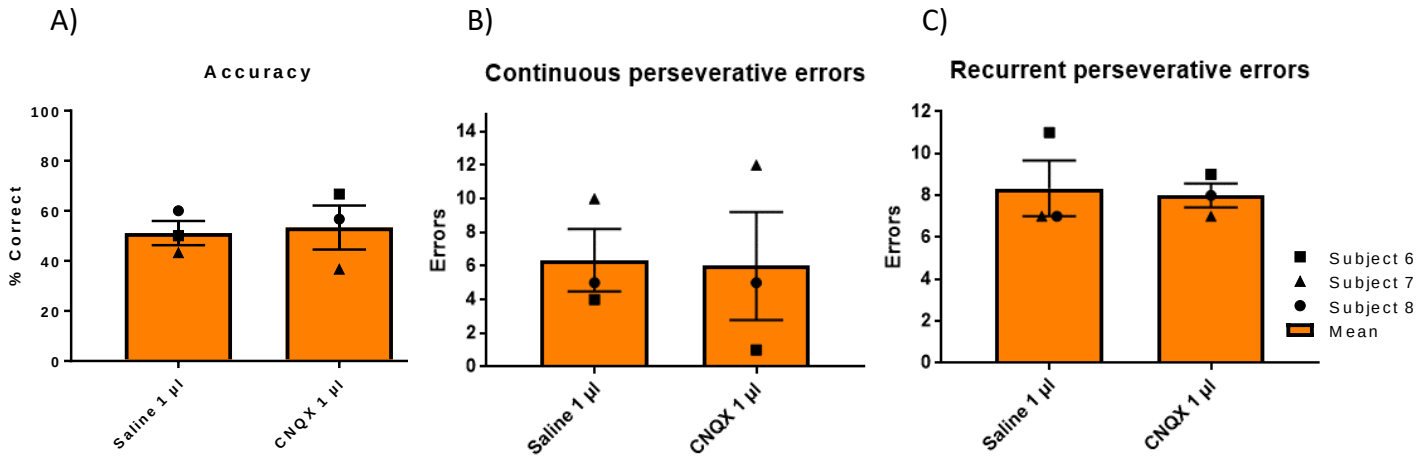


Figure 5.9 Performance of the variable sequencing task after infusion of 1µl CNQX into caudate. The two bars represent the mean for the different manipulations, with individual data-points. Error bars represent standard errors. All graphs have the same key, presented on the far right. A) There was no significant main effect of treatment on accuracy. B) There was no clear effect on continuous perseverative errors. C) There was no clear effect on recurrent perseverative errors.

5.3.5. Variable sequencing task – sulpiride infusions

After having completed all CNQX manipulations subject 6 had three infusions of sulpiride, 10 ng, 60 ng and 600 ng on the variable sequencing task. The subject had no vehicle infusions, but all three doses of the drug had no effect on performance. Due to a lack of vehicle, accuracy on infusion sessions and sessions between infusions is presented for comparison, see **Fig. 5.10**.

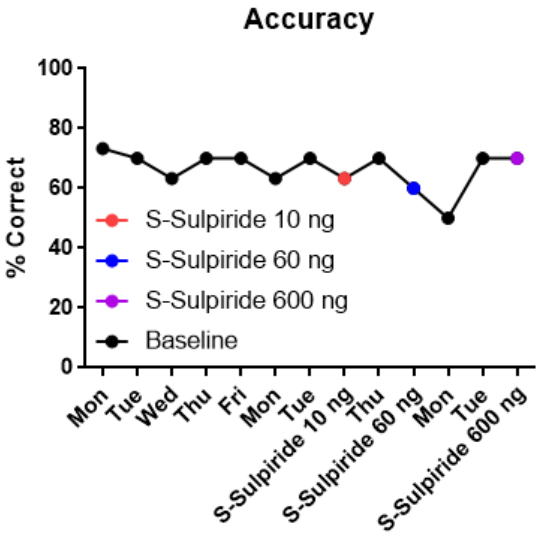


Fig 5.10 Performance of variable sequences following D_2-blockade of caudate. Graph represent baseline data on the number of correct trials per session for subject 6. Each day is labelled with weekday or manipulation. Three different doses of sulpiride were used.

5.4. Discussion

Data have been presented that investigate blockade of glutamatergic input in caudate on variable and fixed spatial response sequences as well as a small pilot study on blockade of caudate D_2-receptors on performance of variable response sequences. No clear effects were observed on performance of fixed or variable response sequences under any manipulations except for a tendency for 0.3 µl infusions of CNQX to decrease the number of perseverative errors.

The studies presented in this chapter contain a low number of subjects and would benefit from at least one additional subject. One additional subject was originally trained and cannulated for these experiments but had to be euthanised after damage to the implant prior to any experimental infusions. Two new subjects were allocated to replace that subject but did not finish training before experiments had to be suspended due to the currently ongoing human Covid-19 pandemic. Infusions of sulpiride were never tested in subjects 7 and 8, as well as on the fixed sequencing task because of the pandemic. It does however need to be emphasised that if these manipulations would have had a similar effect size to the vlPFC manipulations, as presented on these subjects in chapter 3, a significant effect would have been observed.

The only strong effect of CNQX, across tasks and volumes, were on continuous perseverative errors following 0.3 µl infusions of CNQX on the variable sequencing task. Two out of three subjects showed a large decrease in the number of continuous perseverative errors. The third animal barely performed any continuous perseverative errors on baseline and therefore could not show a reduction. Comparing the errors to baseline revealed that for subject 6 and 8, CNQX infusion decreased errors below the 95% confidence interval of baseline. However, this effect still merits further investigations before any firm conclusions can be drawn. The effect was not replicated after infusions of 1 µl CNQX, only one animal still showed a decrease. Indicating either that the animals perform the variable sequencing task differently after having been allowed to heuristically learn a strategy on the fixed sequencing task or that the effect was selective to the area targeted with the small volume, i.e., selective to a small localised area with strong prefrontal inputs almost exclusively from the vlPFC was targeted; but when a wider area was targeted, the effect disappeared. For future subjects, both the 0.3

µl and 1 µl infusions will have to be performed prior to starting the fixed sequence task. If the effect were to be confirmed, it would indicate that blocking input, possibly from the vlPFC, to the caudate would reduce the tendency to repeat immediately preceding responses; suggesting that input to the targeted area of the caudate does not make a positive contribution to performance of variable self-ordered response sequences.

The overall relative lack of effects following CNQX infusions into the caudate were somewhat surprising because it is an important output of the vlPFC which evidently is implicated in the performance of the variable version of this self-ordered task. This lack of involvement could be because the caudate is not engaged during the performance of these tasks, or it could be that CNQX was ineffective as a glutamate receptor antagonist. Neurophysiological studies do however support AMPA-receptors as a target for blocking prefrontal input to the striatum. AMPA-receptors in the striatum are located on the terminals of corticostriatal afferents (Fujiyama et al., 2004), and act to regulate glutamate release through a positive feedback mechanism (Fujiyama et al., 2004; Patel et al., 2001). To this effect, 1 mM CNQX has successfully been used in the dorsal striatum of rats to alter the balance between goal-directed and habitual control of behaviour (Furlong et al., 2014). Also, more relevant, is unpublished findings that control of goal-directed behaviour can be adapted following intra-caudate infusion of 1mM CNQX in the marmoset (Duan et al., manuscript in preparation). NMDA-receptors in the striatum is located on the post-synapses (Fujiyama et al., 2004), and also presented a potential target, however, AMPA-receptor blockade was chosen due to already available behavioural findings supporting an effect. The initial infusion volume of CNQX was very low, to enable precise infusion into an area with strong inputs from the vlPFC. The volume was increased to target a larger area of the caudate but was still without effect. The entire area of the caudate was however not targeted, so it is possible that another area of the anterior caudate was involved in performance. In addition to this, the finding that intra-caudate infusion of sulpiride did not impair performance in a single monkey over a large dose range (although preliminary due to the low n), further strengthens the conclusion that this region of the caudate is not essential for the performance of these tasks, especially considering D_2-r in dorsal striatum is involved in both learning and performance of a skill (Yin et al., 2009). The lowest dose of sulpiride used was in line with effective doses in previous

studies in the rodent striatum (Eagle et al., 2011; Pezze et al., 2007) and should thus have been sufficient to elucidate an effect, if the receptor played a role in performance.

As reviewed in Chapter 1, there is strong evidence for caudate involvement in performance of response sequences in both primates and rodents. However, a discrepancy may exist between task experience in primate studies (Miyachi et al., 2002, 1997). The studies cited above use neural cell-recording and chemical neuromodulation to gain insight into the function caudate plays when animals perform sequences. These animals are experienced in performing sequences, but the actual sequence performed under modulation is new to the animal. In the tasks described here, animals have months of experience in performing the variable sequences, before fully having acquired them and reached a stable baseline for infusions. It is plausible that performance of the variable sequencing task was originally dependent on the targeted area of the caudate, but with experience animals no longer required it for performance. We do however know from Chapter 3 that performance is still dependent on vlPFC. Similar findings have been observed in a serial reversal learning task, where animals after having performed repeated reversals become, rather paradoxically, dependent on putamen for correct flexible performance, as opposed to the caudate (Jackson et al., 2019). Jackson et al also showed that inactivation of the caudate even improved performance. Similarities can be drawn between the reversal improvement and the finding that infusions of 0.3 µl CNQX reduced continuous perseverative errors in the variable sequence task. It is possible that the first few days of the fixed sequencing task would have required caudate for the heuristical-learning of the fixed sequence; however, this was not tested due to the higher variability in performance on the first few days.

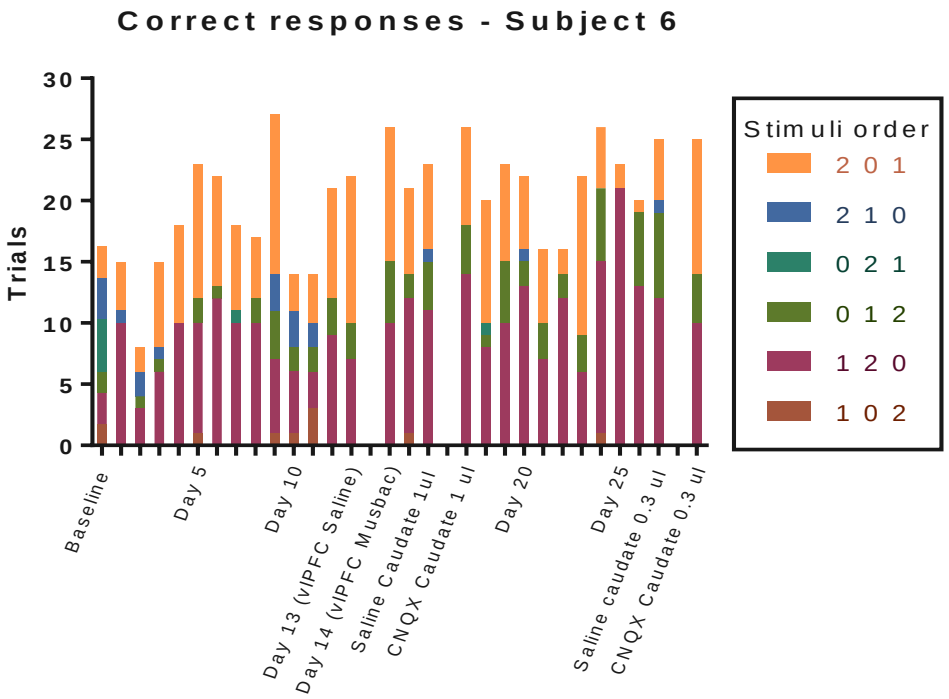
The vlPFC also has projections to the putamen and these could be involved in performance of the fixed or variable sequencing tasks, but the vlPFC could also connect to the putamen indirectly through dense connections with the dlPFC, which in turn also has projections to the caudate and putamen. Investigation into these pathways would be very relevant to our understanding of cognitive deficits in neuropsychiatric disorders, as, for example, disruption in connectivity between caudate/putamen and vlPFC/dlPFC has a direct link to cognitive deficits involving spatial sequencing in OCD patients (Vaghi et al., 2017b).

To summarise, evidence has been presented on the performance of both variable and fixed spatial self-ordered sequences. Rather surprisingly, the data indicates that correct

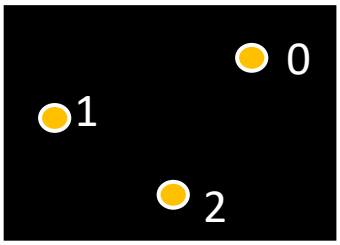
performance of both variable and fixed spatial self-ordered sequences is not dependent on a part of the anterior caudate with inputs from the vlPFC, even though the vlPFC is required for performance of the variable sequences. The findings have been extended by showing preliminary data that D_2-r blockade in the same area of the caudate are without effect on variable response sequencing. The lack of caudate involvement is suggested to be due to the well-trained nature of the task and the putamen is suggested as a target for future investigation.

Supplementary figures

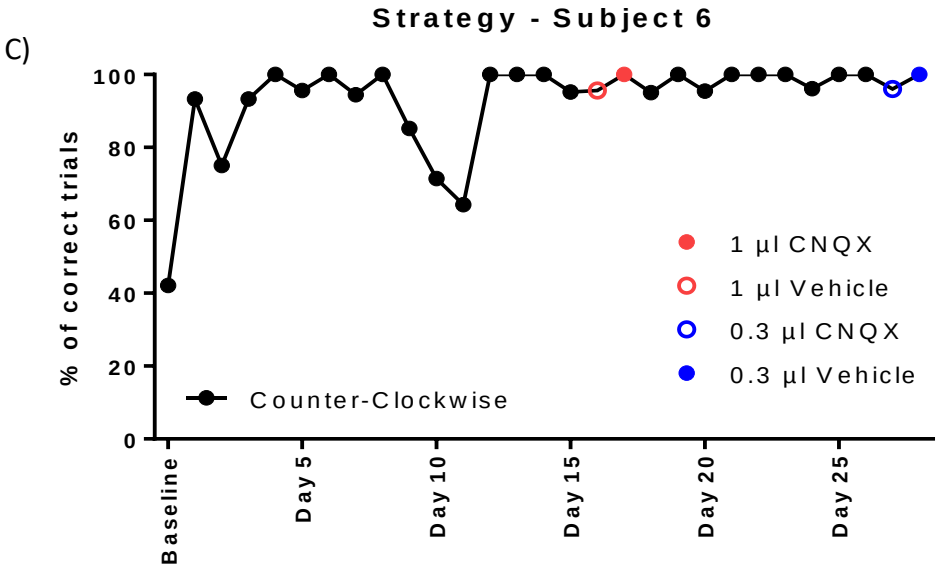
A)



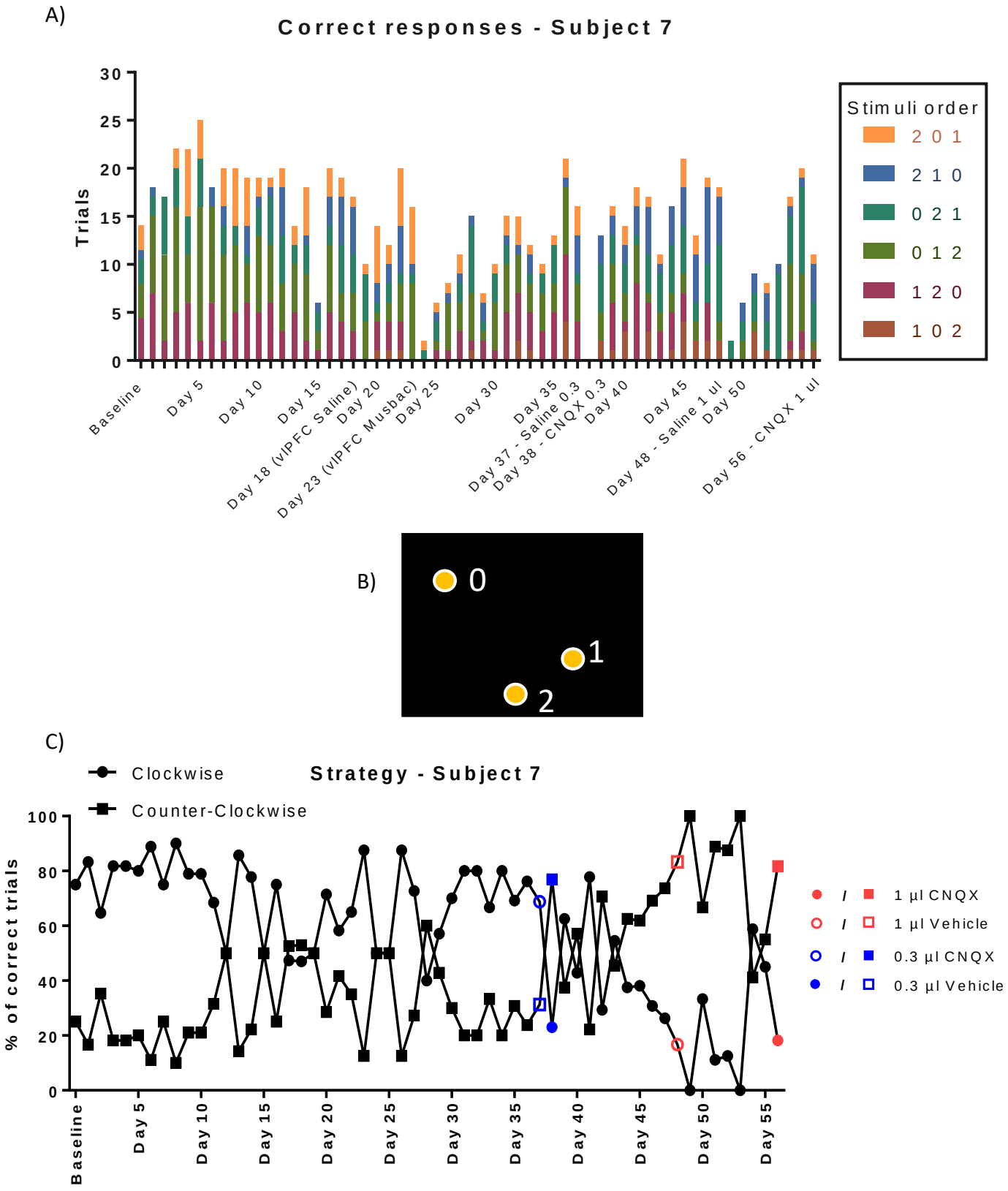
B)



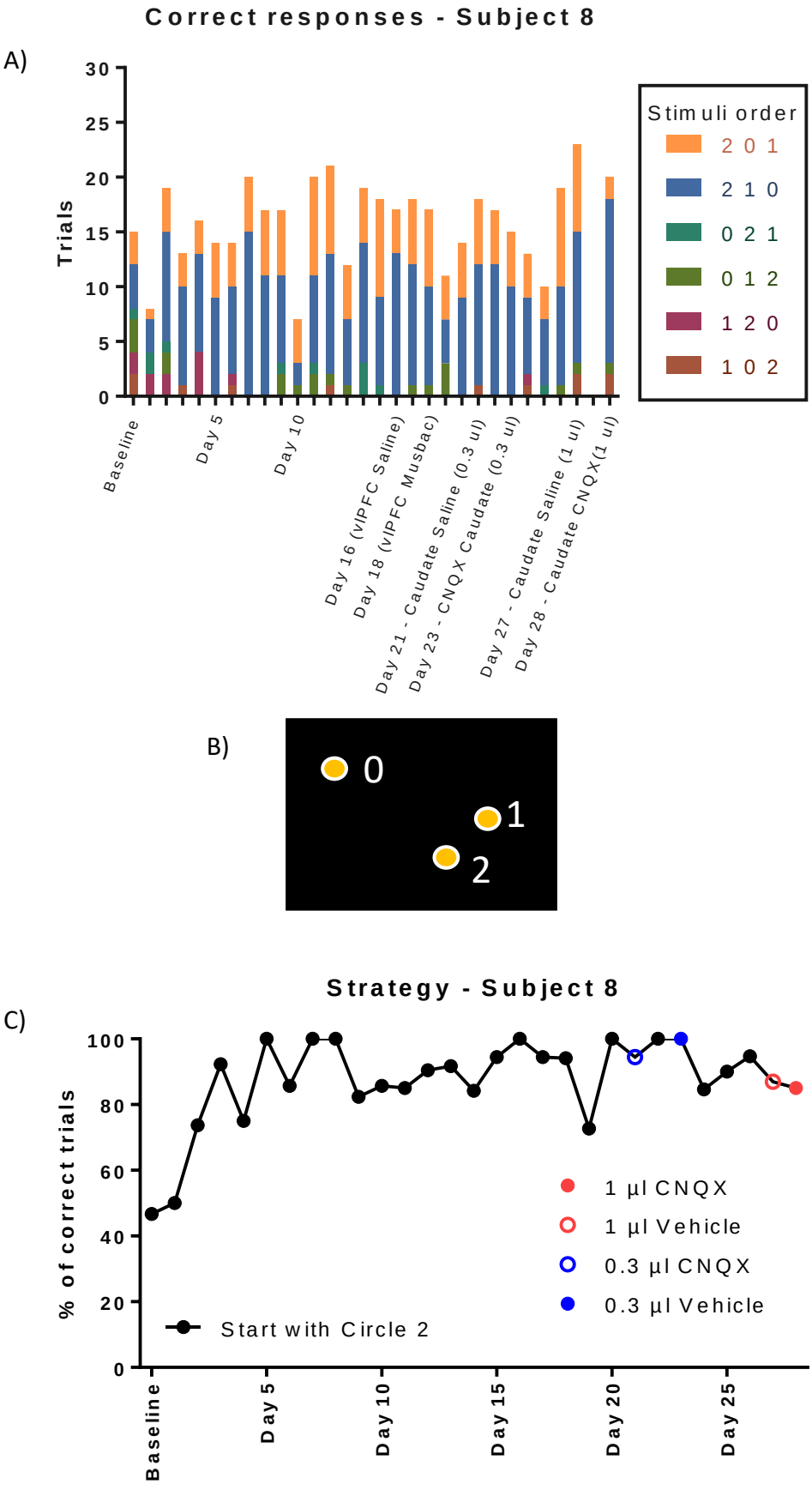
C)



Supplementary figure 5.1. Performance of fixed sequencing task – Subject 6. A) Graph illustrates all correct responses per session on the fixed self-ordered sequencing task. The baseline day represents two months of data, prior to beginning fixed sequencing task, when the fixed sequence was performed at random on the variable sequencing task. Each correct response is represented by colour. Extra gaps between sessions are only to enable easy interpretation of graphs. B) Fixed sequence performed, with stimulus number. C) Graphical representation of percentage of correct trials where the heuristically learned strategy was used.



Supplementary figure 5.2. Performance of fixed sequencing task – Subject 7. A) Graph illustrates all correct responses per session on the fixed self-ordered sequencing task. The baseline day represents two months of data, prior to beginning fixed sequencing task, when the fixed sequence was performed at random on the variable sequencing task. Each correct response is represented by colour. Extra gaps between sessions are only to enable easy interpretation of graphs. B) Fixed sequence performed, with stimulus number. C) Graphical representation of percentage of correct trials where the heuristically learned strategy was used.



Supplementary figure 5.3. Performance of fixed sequencing task – Subject 8. A) Graph illustrates all correct responses per session on the fixed self-ordered sequencing task. The baseline day represents two months of data, prior to beginning fixed sequencing task, when the fixed sequence was performed at random on the variable sequencing task. Each correct response is represented by colour. Extra gaps between sessions are only to enable easy interpretation of graphs. B) Fixed sequence performed, with stimulus number. C) Graphical representation of percentage of correct trials where the heuristically learned strategy was used.

6. General Discussion

6.1. Summary of results

In Chapter 3, investigations were made into the effects of inactivating vlPFC on performance of different spatial self-ordered response sequencing tasks. On the 4-block variable sequencing task, sequences were randomly generated for each trial and the number of stimuli and vanishing times alternated between blocks of trials. Inactivation significantly impaired performance of the most difficult block only, measured as decreased accuracy, increased errors and for recurrent perseverative errors alone. Subjects were also tested in a probe-version of the task, where erroneous responses were not punished, animals could continue responding until all responses had been made. A greater impairment was observed following inactivation in the probe task, as there was an effect of treatment on all blocks of the task. Inactivation decreased accuracy, increased number of errors and the number of errors made on erroneous trials. There was also a significant increase in recurrent perseverative errors, but not continuous perseverative errors. Two further experiments were then performed to investigate the behavioural specificity of these effects. A separate group of animals with identical training performed variable three circle sequences of only one difficulty and the effect of inactivation on accuracy was replicated in this group. These animals then performed a fixed self-ordered sequence and over days of performance animals developed a heuristical strategy for solving the sequence. Inactivation was without effect on fixed sequence accuracy, indicating that vlPFC is required for flexible performance of sequences, but once a heuristic strategy has been developed for a sequence, vlPFC is no longer required for task performance.

In Chapter 4, chemical neuromodulation of vlPFC was investigated on the probe version of the 4-block variable sequencing task, shown to be most dependent on vlPFC. The D_2 receptor antagonist sulpiride and $5HT_{2A}$ antagonist M100907 were infused, separately, into vlPFC and effects of performance were measured. Both antagonists impaired performance but affected different measures. M100907 significantly decreased accuracy and increased errors but did not significantly increase the number of errors on erroneous trials. Sulpiride left accuracy intact but significantly increased the number of errors, by an increase in the number of errors on erroneous trials. No individual error type on its own showed a significant increase after

either infusion. However, there was a trend for M100907 to increase continuous perseverative errors and for sulpiride to increase recurrent perseverative errors.

Finally, in Chapter 5, investigations were made into the wider neural circuitry involved in performance. More specifically, a role of caudate was investigated on performance of both variable and fixed response sequences. The targeted area of the caudate was selected based on primarily receiving prefrontal input from the vlPFC. AMPA-receptor antagonist CNQX was infused into the area to block pyramidal input. The infusions revealed no significant effects on the fixed or variable sequencing task. However, there was an ambiguous effect on the variable sequencing task. On the variable sequencing task two out of three animals showed a strong reduction in continuous perseverative errors, this was however not significant on a group level. The third animal performed almost no baseline errors and could thus not show a reduction.

Collectively, these findings clarify the role of vlPFC in goal-directed control of behaviour and extends the limited understanding of its neuromodulation. It also builds on the foundation of knowledge about how sequential behaviour is organised. Prior to these experiments, a role for vlPFC in organising self-ordered sequential behaviour had only been shown in neuroimaging studies or excitotoxic lesion studies. Similarly, the behavioural specificity was not clear. Very few studies have investigated specific chemical neuromodulation of vlPFC, and it had never been investigated on a sequencing task. The findings that blockade of pyramidal input to the caudate does not impair task performance were unexpected but serves as important evidence for understanding the neural circuitry involved in sequencing performance. However, it needs to be acknowledged that the current experiments are unable to confirm the efficacy of CNQX at blocking transmission.

6.2. Inactivation of vlPFC, behavioural specificity and use of strategy

A broad role for vlPFC in regulation of cognition and working memory was presented in the introduction. vlPFC is required for optimal performance of visual discrimination tasks, such as attentional set shifting (Dias et al., 1996), reversal learning with novel stimuli (Rygula et al., 2010) and visuomotor association tasks (Bussey et al., 2001; Puig and Miller, 2012). A role in visual discrimination have been extended to show that vlPFC impairs use of behavioural strategies in these tasks (Baxter et al., 2009; Bussey et al., 2001). A role for vlPFC is also

evident in working memory tasks which require organisation of behaviour (Collins et al., 1998; Owen et al., 1996; Walker et al., 2009a), however, vlPFC is not required to maintain information 'online' during delay in a match to sample task (Rushworth et al., 1997). Nonetheless, two important studies in primates, which investigated vlPFC in performance of sequences had a global delay component after each response (Collins et al., 1998; Walker et al., 2009a). The task used in this thesis were designed to assess the role of vlPFC in behavioural planning with minimal working memory load. The finding that inactivation of vlPFC impaired task performance, support the hypothesis that vlPFC helps to organise sequential behaviour, as opposed to merely maintaining information during a delay.

To summarise findings, it has been demonstrated that vlPFC is required for judgment of what visual dimension of a stimulus needs to be attended to, establishing reward associations between two novel stimuli and associate visual stimuli with motor responses. The function also extends to organising response for maximising reward, either responses that needs to be temporally associated (Baxter et al., 2009) or abstract rules such as 'lose-stay' (Bussey et al., 2001). As demonstrated in this thesis, vlPFC behavioural planning also extends to performance of self-ordered spatial sequences. These findings can likely be harmonised in a suggested role for vlPFC in active (strategic) retrieval (Petrides et al., 1996).

The finding that vlPFC inactivation only impaired performance of variable but not fixed self-ordered sequences indicate a role in goal-directed control of behaviour, when it requires constant updating and behavioural strategies cannot (easily) be reduced into motor sequences. However, once a heuristic strategy has been learnt as a procedural skill, vlPFC is no longer required for updating a behavioural strategy and thus inactivation is without effect. This resembles findings from reversal learning (Rygula et al., 2010) and visuomotor learning (Puig and Miller, 2015, 2012), which demonstrated that vlPFC were only required for performance during learning, but did not affect already familiar associations.

For all studies in this thesis, the number of continuous and recurrent perseverative errors have been presented separately. It is however at this stage not clear how these two varieties of perseveration differ from each other and also the behavioural relevance of respective error type. The significant effect of increased recurrent, but not continuous perseverative errors after inactivation does however contrast with the near significant effect of increased continuous perseverative errors following infusion of m100907. Indicating that it might be

possible for different modulations to produce separable error phenotypes. It is difficult to compare errors from these experiments with demonstrated impairments in previous sequencing tasks in humans and primates, as almost no studies present which errors were performed. Even though the different errors cannot clearly be accounted for in this thesis, they are presented separately to enable comparisons for future studies.

The findings from Chapter 3 demonstrate the utility of the study design to investigate the role of vlPFC in sequential behaviour. However, one limitation to the variable sequencing tasks used in this thesis is the lack of a strategy measure. All attempts at creating such a measure for the variable sequencing task were inconclusive. Previous studies that have measured behavioural strategies on this task (albeit with delay) all used four stimuli trials (Collins et al., 1998; Taffe and Taffe, 2011). Three stimuli trials limit the number of possible correct responses per sequence to six and many responses become symmetrical, making it difficult to distinguish between them and thus difficult to classify them. Four sequence trials require longer sequences and thus also increasing the requirement to organise behaviour. On 1-block tasks, animals reached a stable baseline faster and could potentially be trained to perform 4 block trials without an extensive extra period of training. This would potentially be beneficial in further experiments, particularly to explore the role of vlPFC further but also to contrast with future causal investigations into other areas.

6.3. Neuromodulation

In Chapter 4, the effect of $5HT_{2A}$ and D_2 receptor blockade were investigated on performance of variable response sequences. Both drugs impaired performance by increasing errors but caused different profiles of impairment.

Neurons containing D_2 receptors in dlPFC demonstrate activity related to responses during an oculo-motor working memory task (Wang and Goldman-Rakic, 2004). Activity not dissimilar have also been found in the vlPFC (Puig and Miller, 2015). This neuronal firing is thought to reflect processing of motor related feedback (Wang and Goldman-Rakic, 2004) and impairments in this processing have been suggested to cause impairments in correcting

behaviour (Arnsten et al., 2015). D_2 receptors are primarily found on pyramidal cells in layer V of PFC (Lidow et al., 1998) and could thus be hypothesised to serve a role in monitoring feedback from subcortical areas. When required, they can inhibit pyramidal output to stop current behaviour and thus a new behavioural plan can be generated, illustrated in **Fig. 6.1**. The findings from Chapter 4 are generally in agreement with this finding, based on a significant increase in errors per erroneous trials, without a significant decrease in accuracy.

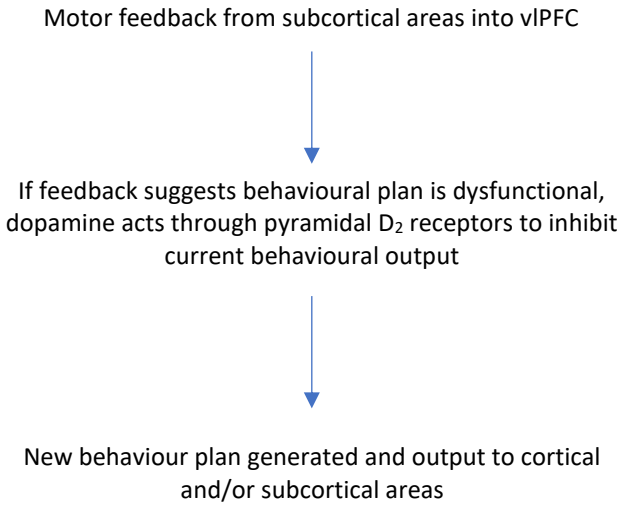


Figure 6.1 Proposed schematic for intra-vlPFC D_2 receptors in response sequencing

The role $5HT_{2A}$ receptors play in the lPFC is less clear, but it has been shown that $5HT_{2A}$ receptor blockade in dlPFC modulate spatial tuning of cells during a working memory task (Williams et al., 2002). These effects were suggested to indicate a role in alternating the signal-to-noise ratio of sensory inputs and blockade of $5HT_{2A}$ was suggested to impair attentional allocation. Pyramidal $5HT_{2A}$ receptors in the lPFC are located on the apical dendrite (Jakab and Goldman-Rakic, 1998) and has been suggested to amplify excitatory synaptic currents (Marek and Aghajanian, 1999). As presented in the Introduction, there is strong interplay between $5HT_{2A}$ and $5HT_{1A}$ receptors in the PFC, through both pyramidal cells and interneurons. $5HT_{1A}$ receptors are inhibitory on the pyramidal axon, and without excitatory mechanisms by $5HT_{2A}$, would further contribute to pyramidal cell inhibition. Based on these findings, I hypothesize that the impairment seen following infusion of $5HT_{2A}$ antagonist into the vlPFC are a result of pyramidal $5HT_{2A}$ receptors being unable to amplify

task relevant sensory inputs, used to encode a behavioural plan, see the schematic illustrated in **Fig. 6.2**. However, dopaminergic error-correction mechanisms might still be intact. The findings that accuracy were significantly impaired, but ability to correct erroneous responses were not significantly impaired, are consistent with this proposal.

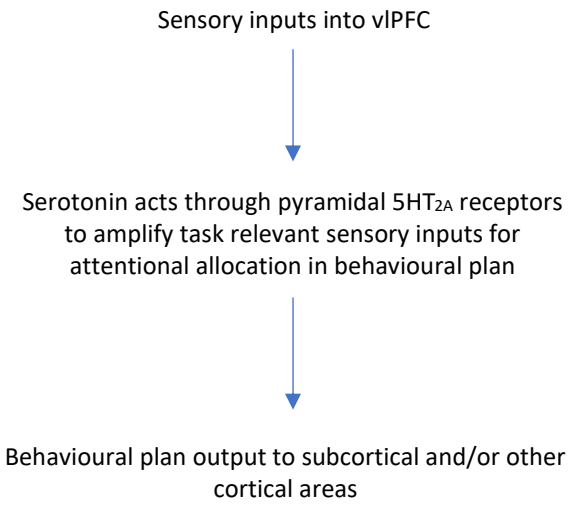


Figure 6.2 Proposed schematic for intra-vlPFC $5HT_{2A}$ receptors in response sequencing

However, it needs to be emphasized that the drug treatments were not completely dissociable, even though they caused different significant impairments. The slightly interweaved effects could potentially be explained by co-expression of these receptors on neurons in layer V, unfortunately, to my knowledge, no study has investigated co-expression of these receptors in PFC. Another plausible explanation might be related to the $5HT_{1A}$ receptor. Atypical antipsychotics that act to blockade both D_2 and $5HT_{2A}$ receptors have been shown to mediate part of their effects through a $5HT_{1A}$ mechanism, regardless of intrinsic affinity to the receptor (Ichikawa et al., 2001).

Eighty percent of pyramidal neurons that express either $5HT_{2A}$ receptors also express $5HT_{1A}$ receptors, it would therefore be valuable to investigate the effect of local infusion of $5HT_{1A}$ agonist in the vlPFC on performance of variable sequences. Due to the high co-expression on pyramidal cells, it could be theorised that $5HT_{1A}$ agonists would cause a similar impairment as $5HT_{2A}$ antagonist. However, the effect observed following $5HT_{2A}$ blockade cannot be selectively attributed to either pyramidal or interneurons with current techniques. Chemogenetic techniques could allow for these cell types to be studied separately and would represent important future steps to our understanding of vlPFC chemical neuromodulation. Both $5HT_{2A}$ and $5HT_{1A}$ are located in deep layers of the PFC, while ionotropic $5HT_3$ receptors are expressed in slow spiking interneurons in superficial layers of monkey PFC (Jakab and Goldman-Rakic, 2000) and would thus present another future target for understanding serotonergic modulation of goal-directed behaviour.

Further investigation into dopaminergic manipulations would also be beneficial. Particularly studying a role for prefrontal D_1 receptors would be of interest. It has been suggested that D_1 act to maintain information, while D_2 act to switch between behavioural states (Durstewitz and Seamans, 2008). It is thus hard to predict how stimulation or blockade of the D_1 receptor would affect self-ordered response sequencing. However, selective blockade of vlPFC D_1 has been shown to impair performance of visuomotor associations (Puig and Miller, 2012) so a role in flexible behaviour has been demonstrated, but it is unknown if this role would extend to sequencing. Prefrontal D_4 receptors have been demonstrated to play a role in set-shifting in rats (Floresco et al., 2006). They showed that D_4 receptor stimulation impaired performance, while, more interestingly, D_4 blockade improved performance. This effect is probably mediated through mechanisms on NMDA-receptors (Jardemark et al., 2005; Wang et al., 2003). D_4 receptors might therefore be a good candidate for investigations, as efforts to find chemical neuromodulators that can improve cognitive performance are key to the development of new pharmacological agents to treat psychiatric disorders, discussed later in this chapter.

A role for both prefrontal serotonin and dopamine in performance of response sequences has thus been advanced, although this is in contrast to previous findings that depletion of serotonin (Walker et al., 2009a) and dopamine (Collins et al., 1998) are without effect on self-ordered sequencing performance. The tasks they used are very similar to the one used in this thesis, except for a global delay after each response in the depletion studies. However, the dopamine depleted group showed impairments in spatial delayed response, but no impairments in sequencing, indicating that global delay is unlikely to explain the differences. A potential explanation could be that the depletions were not total, however it could also be related to compensatory mechanisms or opposed receptor mechanisms cancelling each other out. There is no way to tell from the current study, but it emphasises the importance of confirming findings from depletion studies with more acute methods of chemical modulation.

6.4. Neural Circuitry

This section suggests further studies required for understanding the wider neural circuitry involved in performance, illustrated in **Fig. 6.3**. In the introduction a role was presented for basal ganglia in performance of sequences in both rodents and primates. Particularly, in the

light of findings that inactivation of anterior caudate and putamen impair “early” sequences in the 2x5 task (Miyachi et al., 1997), it is surprising that CNQX infusion into the caudate did not impair performance of variable or fixed response sequences. As discussed in chapter 5, it is unlikely that the null effect on accuracy is related to the lack of efficacy of the drug, but it is by no means out of the question. Future experiments could try an NDMA receptor antagonist or GABA receptor agonists. However, more likely is that the lack of effect is related to the well-trained nature of the task, even though behaviour is still required to be flexible. Similar results have been found in a serial reversal learning task, where putamen and not caudate were important for flexible performance (Jackson et al., 2019). Due to one animal performing almost no continuous perseverative errors on baseline, the study requires more subjects to explore a potential role in the regulation of continuous perseverative responding. However, once that potential role has been established, several other investigations would be of interest. Further experiments should aim to understand the role of fronto-striatal circuits in sequencing; dlPFC (area 46) would be a priority target in the prefrontal cortex and

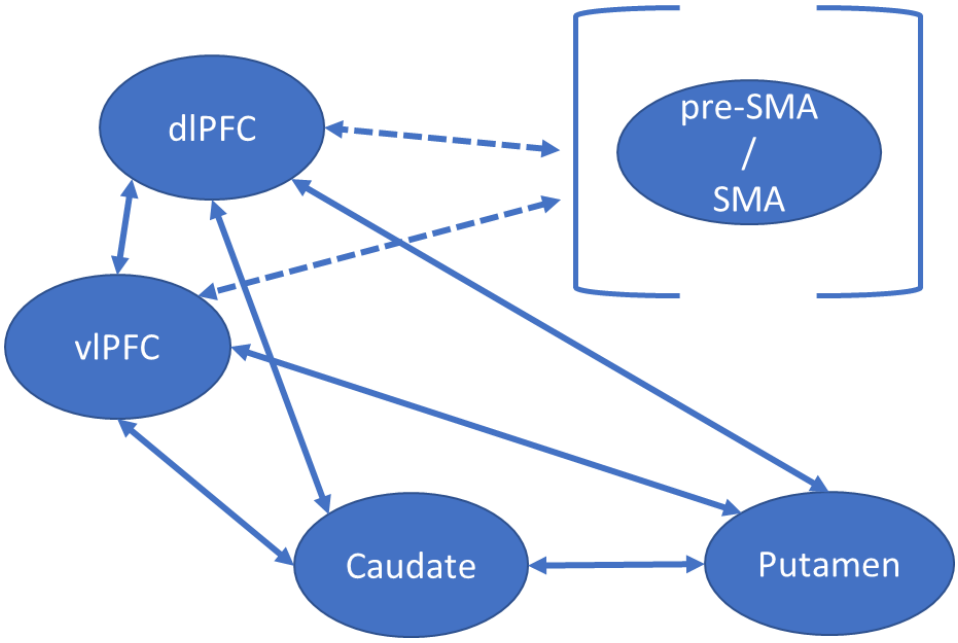


Figure 6.3 Key brain regions requiring causal investigations for understanding the wider neural network on performance of self-ordered sequences. A causal role of vlPFC was presented in this thesis. Further investigations into the causal role of dlPFC is required. The role of caudate needs further clarification and putamen needs to be causally investigated. After the causal role of each area has been established, each circuit could be investigated in isolation using chemogenetic techniques. Further to this, causal investigations into the role for pre-SMA and SMA would also be beneficial, particularly if a no clear role for basal ganglia can be established.

putamen would be a priority target in the basal ganglia. Of interest would also be infusions into a more anterior area of the caudate, to which the dlPFC projects. vlPFC and dlPFC are highly interconnected (Petrides and Pandya, 2002, 1999; Roberts et al., 2007) and both areas have been demonstrated to show increased activity in performance of the CANTAB SWM which similarly involves response sequencing (Owen et al., 1996). The areas do however perform different functions, as demonstrated in the same study showing that only vlPFC increased activity during the spatial span task, while only dlPFC increased activity for the spatial monitoring task. A first important step would be to investigate the causal role of dlPFC in performance of both the variable, and fixed sequencing task. Similar investigations into the role of putamen, and more anterior parts of caudate will be important for understanding this network. Future developments of chemogenetic techniques in primates could allow investigations into these circuits separately. For example, vlPFC-dlPFC or vlPFC-putamen circuits could specifically be inhibited or stimulated, and the role of these circuits could be investigated in isolation. This would be particularly relevant for disorders with sequencing deficits, as specific disorder-relevant circuits could be selectively modulated.

In the introduction, evidence was also presented for a role of cortical motor areas in planning/executing the motor actions of a behavioural sequence. Neural activity in motor area M1 reflect motor actions originating from lPFC behavioural plans (Mushiake et al., 2006) and cells in the SMA and pre-SMA have been demonstrated to reflect individual motor components of a sequence (Clower and Alexander, 1998), and for SMA, how these movements relate to each other (Tanji and Shima, 1994). The marmoset area 6m has been suggested to contain two regions, homologous to SMA and pre-SMA (Bakola et al., 2015; Burman et al., 2014). vlPFC has no (or very restricted) connectivity with M1 (Burman et al., 2014), but does connect to area 6m (Roberts et al., 2007). Thus, connectivity between SMA or pre-SMA and vlPFC could be a relevant circuit for understanding the impairment following vlPFC inactivation. Investigations into these circuits would have less relevance for psychiatric disorders OCD (Dong et al., 2020) and schizophrenia (Zhou et al., 2007), but might be more relevant for understanding sequencing deficits in Parkinson's disease (Catalan et al., 1999; Wilkinson et al., 2009). Investigating the possible role of area 6m on the tasks presented in this chapter could be important for determining the validity of these tasks for translational studies that aim to investigate impaired behavioural planning in disease. Studies on this area

will be particularly relevant if follow up studies cannot establish a clear role for the basal ganglia.

6.5. Relevance for Schizophrenia and OCD

Patients suffering from OCD and schizophrenia show impairments across a range of sequencing tasks. In the introduction evidence was presented that demonstrate an impairment in CANTAB SWM, CANTAB SOC, CANTAB OTS and a sequence generation task.

For OCD, planning deficits are correlated with decreased resting state connectivity between dlPFC and putamen, while deficits in cognitive flexibility were correlated with resting state connectivity between vlPFC and caudate (Vaghi et al., 2017b). Decreased functional connectivity between dlPFC and putamen during performance of CANTAB OTS has also been demonstrated in OCD patients and first-degree relatives, as opposed to healthy controls (Vaghi et al., 2017a), extending previously observed findings to an endophenotype of the disorder. For schizophrenia, there has similarly been studies showing dysconnectivity between lPFC and basal ganglia (Zhou et al., 2007), dysconnectivity in these circuits are also related to poor executive functioning (Quidé et al., 2013). Cognitive deficits have been suggested to strongly represent liability to schizophrenia, as opposed to schizophrenia causing impaired cognition (Toulopoulou et al., 2019, 2015). These cognitive deficits are not limited to sequencing tasks. However, a recent study demonstrated that performance of CANTAB SWM, OTS and spatial span are heritable traits associated with schizophrenia and that impairments in OTS and spatial span extend beyond the reductions in IQ that occur in schizophrenia (Lemvigh et al., 2020). It is thus of utmost importance to understand how sequencing behaviour is controlled and its neurochemical modulation.

The studies presented in this thesis demonstrate a causal role of vlPFC in organising behaviour for performance of variable response sequences and thus; these tasks represent a method for assessing vlPFC dependent sequencing behaviour, impaired in OCD and schizophrenia. A strong advantage of the methodology involving microinfusions, as opposed to previously utilised permanent lesions, is that specific neuromodulation can also be investigated. The ability to make localised infusions opens up opportunities for investigating novel and already available drugs, within circuits, of relevance for cognitive deficits in psychiatric disorders. Two drugs targeting different receptors, were tested on performance of variable self-ordered

response sequences. Infusion of $5HT_{2A}$ receptor antagonist M100907 impaired accuracy of the task, as compared to vehicle, while D_2 antagonist sulpiride left accuracy intact, but increased errors through impaired error correction.

There is indeed support for an involvement of these receptors in these psychiatric disorders as genetic polymorphisms of both these receptors have been observed in patients suffering from schizophrenia (Blasi et al., 2015; Üçok et al., 2007) and OCD (Denys et al., 2006; Taylor, 2013). OCD-patients also show downregulation of both D_2 and $5HT_{2A}$ receptors (Moresco et al., 2007; Perani et al., 2008). The evidence for D_2 and $5HT_{2A}$ receptors in schizophrenia is clear, as antipsychotics, which targets these receptors, are first line treatment for schizophrenia. However, they do not treat cognitive deficits well (Mishara and Goldberg, 2004). Recovery of cognitive functioning is important for long-term community outcome (Green et al., 2004, 2000), and in particular restored performance of action sequences (Semkovska et al., 2004). The findings that (although local and not systemic) treatment with $5HT_{2A}$ or D_2 antagonists impaired these measurements are troubling, as it indicates that current treatments for schizophrenia might impede successful community outcomes. Indeed, there is evidence in support of this. Patients treated with antipsychotics with high affinity to the $5HT_{2A}$ receptor show decremental planning performance over time (Tyson et al., 2004b). There is also evidence from healthy individuals that systemic treatment with sulpiride impairs sequencing performance (Mehta et al., 1999). The findings in this thesis therefore present novel evidence that vlPFC might represent a neural locus for these effects. The current psychopharmacological findings are of less immediate clinical relevance for OCD at this stage, but importantly suggest that these receptors might be implicated in the neuropsychopathology of the disorder. Findings that $5HT_{2A}$ blockade impair sequencing performance is of particular interest for OCD; as it has been observed that drug-naïve OCD patients show reduced $5HT_{2A}$ receptor availability within the lPFC (Perani et al., 2008), indicating that the reduction of these receptors might be a root cause for the impairment in behavioural planning. Future investigations into the chemical neuromodulation of vlPFC, using the current task design, present a method for evaluation of novel pharmaceuticals which could improve functional outcome for schizophrenia patients and provide symptom relief and recovery for OCD patients.

Future investigations on the neural circuitry, as suggested above, present an opportunity to not only investigate the causal role of single neural regions implicated in disease, but to study the role of specific neural circuits in performance of action sequences. This may help to create pre-clinical models that allow studies into endophenotypes of psychiatric disorders. With increased information about how these circuits operate, the understanding of how sequential behaviour is controlled could provide important tools for the development of new psychological, as well as new pharmacological, treatments.

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